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(54) Title: DEPIGMENTING ACTIVITY OF AGOUTI SIGNAL PROTEIN AND PEPTIDES THEREOF

(57) Abstract

The invention is an agouti signaling protein and peptides as well as pharmaceutical compositions thereof and their use in methods of inhibiting melanin production by melanocytes. The agouti signaling protein and peptides thereof are useful in cosmetics and in clinical prevention and treatment of hyperpigmentary conditions. Methods for screening peptides for melanogenesis inhibiting activity are also provided.

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DEPIGMENTING ACTIVITY OF AGOUTI
SIGNAL PROTEIN AND PEPTIDES THEREOF

Background Of The Invention

Mammalian melanocytes can produce two types of melanin, eumelanin (which is black and/or brown in color) and pheomelanin (which is red and/or yellow in color) (Prota, 1992; Ito, 1993a). Switching between these two types of melanins in

5 follicular (i.e. hair bulb) melanocytes elicits a temporary shift from eu- to pheomelanogenesis, which is responsible for the wild-type agouti pigment pattern of murine hair color; i.e. a yellow striped band against a black background on each hair shaft, as reviewed by Silvers (1979) and Hirobe (1991). This physiological switch is controlled by the *agouti* locus, which has recently been cloned (Bultman et al., 1992;

10 Miller et al., 1993). The *agouti* locus-encoded protein is thought to be secreted by nonmelanocytic follicular cells (Silvers, 1958) and functions as an antagonist of the melanocyte-stimulating hormone (αMSH) receptor, which is expressed specifically by melanocytes (Lu et al., 1994). The recessive black mutation (nonagouti; a) at the *agouti* locus results in a nearly complete loss of *agouti* RNA which causes the constitutive

15 production of eumelanin black hairs. In contrast, the dominant lethal yellow mutation (Ay/a) elicits the ubiquitous ectopic production of *agouti* RNA in nearly all tissues of the body and throughout the entire phase of the hair growth cycle, prompting the production of completely yellow pheomelanic hairs (Ito and Fujita, 1985; Duhl et al., 1994a,b).

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For many decades, melanosomal proteins that regulate melanin biosynthesis have been studied and characterized, especially those required for eumelanogenesis, as reviewed by Hearing and Tsukamoto (1991) and Hearing and King (1993). Tyrosine (EC 1.14.18.1), which is encoded at the *albino* locus, is the essential

25 enzymatic protein for both types of melanin formation. Tyrosinase is a trifunctional enzyme with three catalytic activities: tyrosine to 3,4-dihydroxyphenyl-alanine (DOPA), the oxidation of DOPA to dopaquinone and the oxidation of 5,6-dihydroxindole (DHI) to indole-5,6-quinone (Korner and Pawelek, 1982; Hearing, 1987; Tripathi et al., 1992).

Other tyrosine-related proteins (TRP) have been shown to regulate eumelanogenesis catalytically at steps distal to tyrosinase. TRP1, encoded at the *brown* locus, functions as 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase (Jimenez-Cervantes et al., 1994; Kobayashi et al., 1994b) while TRP2, encoded at the *slaty* locus, functions as

5   DOPAchrome tautomerase (EC5.3.2.3) (Barber et al., 1984; Aroca et al., 1990; Tsukamoto et al., 1992; Jackson et al., 1992). The *silver* locus-encoded protein had been proposed to function in melanogenesis catalytically within the melanosome, and although it has some limited homology to the tyrosinase-related proteins (Kwon et al., 1991), it has been recently demonstrated to be a melanosomal matrix protein and to

10   have none of the known melanogenic activities (Zhou et al., 1994; Kobayashi et al., 1994a). The product of the *pink-eyed dilution* locus is also a melanosomal protein that actively participates in the regulation of melanogenesis (Tamate et al., 1989; Chiu et al., 1993; Rosemblat et al., 1994).

15   During pheomelanogenesis, the activity and expression of tyrosinase has been reported to be lower than that found during eumelanogenesis (Barber et al., 1985; Burchill et al, 1986, 1989; Lamoreux et al, 1986; Movaghar and Hunt, 1987; Tamate et al., 1989; Granholm et al., 1990; Kappenman et al., 1992). In addition to tyrosinase, thiols are essential to capture the dopaquinone made enzymatically by tyrosinase in order

20   to produce the cysteinyldopas necessary for pheomelanogenesis (Figure 1). Subsequent cyclization and polymerization of cysteinyldopas in an uncharacterized series of reactions results in the production of the high molecular mass complex known as pheomelanin (Prota, 1992; Hearing and King, 1993; Ito, 1993a). The switch between eu- and pheomelanogenesis has been proposed to be regulated enzymatically primarily at

25   the level of tyrosinase (Ito, 1993a). The potential roles of other melanogenic gene products during pheomelanogenesis, however, remain unclear, since there have been few studies about the expression and function of such proteins during pheomelanogenesis. To date, only the absence of DOPAchrome tautomerase activity in yellow mice has been reported (Barber et al., 1985), as has the absence of TRP1 mRNA expression in

pheomelanogenic mice (Thody and Burchill, 1992) and human melanoma cells (Del-Marmol et al, 1993).

The present invention determines the transcriptional and translational
5   levels of the expression and catalytic functions of tyrosinase, TRP1, TRP2 and the silver protein during pheomelanogenesis. The expression and melanogenic activities of those proteins in hair bulbs of wild type agouti mice during their pheomelanogenic phase is also determined in the present invention. The present invention shows that TRP1, TRP2 and the silver protein function specifically in eumelanogenesis and may play an important
10   role in the production of eumelanosomes. The down-regulation of expression of those proteins during melanogenesis is shown in the present invention using agouti signaling protein.

Summary Of The Invention

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The present invention is a biologically active peptide of the Agouti Signaling Protein which has depigmenting activity.

The present invention is a method of down-regulating one or more
20   melanogenic enzymes involved in melanin synthesis.

The present invention is also the use of the Agouti signaling protein and biologically active peptides thereof in methods of inhibiting melanin synthesis.

25   A further aspect of the invention is the treatment of hyperpigmentary conditions and diseases using an effective amount of agouti signaling protein or peptides thereof.

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Another aspect of the invention is a pharmaceutical composition of agouti signaling protein or biologically active peptides thereof and a pharmaceutically acceptable carrier.

5   The present invention is also a method for screening for biologically active peptides of the agouti signaling protein and other compounds useful in inhibiting melanin synthesis.

Brief Description Of The Figures

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These and other objects, features, and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

15   Figure 1 shows the eumelanin and pheomelanin biosynthetic pathway.

Figure 2 shows analysis of melanogenic proteins expressed by hair bulb melanocytes of newborn lethal yellow and black mice. A 20 μg sample of protein from extracts of hair bulbs of 10 day newborn lethal yellow or black mice (or melan-a cells)
20   were electrophoresed in SDS/polyacrylamide gels, transferred to membranes and analyzed for melanogenic proteins by western immunoblotting, as detailed in Materials and Methods. Skin tissues in organ culture (or melan-a cells) were labeled with [^{35}S]methionine for 4 hours, solubilized and immunoprecipitated by antibodies noted, separated by SDS-PAGE, dried and fluorographed, as detailed in Materials and
25   Methods.

Figure 3 shows Northern blot analysis of RNAs present in hair bulb melanocytes of newborn lethal yellow, black and agouti mice. mRNAs from hair bulbs of 10 day newborn lethal yellow, black and agouti mice were isolated and

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electrophoresed (2 μg/lane), transferred to nylon membranes and repeatedly hybridized
with ^{32}P-labeled probes for tyrosinase, TRP1, TRP2 and silver protein or GAPDH
(glyceraldehyde-3-phosphate dehydrogenase), as detailed in Materials and Methods. s,
Svedberg units.

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Figure 4 shows expression of melanogenic proteins in regenerating hair
bulbs of lethal yellow and black mice. Anagen growth hairs were induced by plucking
the dorsal telogen hairs of 2-month-old sibling lethal yellow and black mice. Seven 1 cm
x 1 cm squares on each mouse were randomly plucked at 0 (immediately before), 2, 4, 6,
10   8, 10 and 12 days before sacrifice. Dorsal skin samples were collected from 3 mice of
each genotype, solubilized and analyzed by western immunoblotting, as detailed for Fig.
2; 30 μg protein was separated in each lane.

Figure 5 shows expression of melanogenic proteins in dorsal skins of
15   newborn agouti (A/A) mice. Dorsal skins were collected from 3, 5, 7, 9 and 11 day
newborn agouti mice, solubilized and analyzed by western immunoblotting, as detailed
for Fig. 2; 30 μg protein of each dorsal skin extract (10 μg of the melan-a cell extract)
were separated in each lane.

20   Figures 6A and 6B show expression of melanogenic proteins of dorsal
skins of agouti (A/a) and black (a/a) mice. Expression of melanogenic proteins in dorsal
skins of 5 and 11 day newborn sibling agouti and black mice was examined using
western immunoblotting, and metabolic labeling and immunoprecipitation, as detailed in
Materials and Methods. (Figure 6A) Proteins in extracts of dorsal skins of agouti and
25   black mice were solubilized and analyzed by western immunoblotting, as detailed for
Fig. 2; 40 μg protein of each dorsal skin extract (10 μg of the melan-a cell extract) was
separated in each lane. (Figure 6B) Skin tissues in organ culture was labeled with
[^{35}S]methionine for 6 hours, solubilized and analyzed by immunoprecipitation, as
detailed in Materials and Methods.

6

Figures 7A and 7B show the Northern blot analysis of RNA levels of tyrosinase and GAPDH from melan-a cells cultured in the presence of various concentrations of agouti protein (Fig. 7A) and cultured in the presence of 10nM of agouti protein for 1, 2 or 4 days (Fig. 7B).

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Figures 8A and 8B show the ultrastructure of melan-a cells grown for five days in the presence of 10nM agouti protein (Fig. 8B) or in the absence of agouti protein (Fig. 8A) (mag x8000).

10   Figure 9 shows the Northern blot analysis of RNA levels of tyrosinase, TRP 1, TRP 2, MSH-R, and GAPDH from melan-a cells cultured 1 day in the presence of various concentrations of agouti protein or in the absence of agouti protein.

Figure 10 shows the Northern blot analysis of RNA levels of tyrosinase,
15   TRP 1, TRP 2, MSH-R and GAPDH from melan-a cells cultured 5 days in the presence or absence of 10nM MSH, in the presence or absence of 10nM agouti protein, or in the presence of both MSH and agouti protein.

Figure 11 shows the results of metabolic labeling of immunoprecipitation
20   of tyrosinase, TRP 1 and TRP 2 protein from melan-a cells cultured 5 days in the presence or absence of 10nM MSH, in the presence or absence of 10nM agouti protein, or in the presence of both MSH and agouti protein.

Figure 12 shows the Western blot of tyrosinase, TRP 1 and TRP 2
25   protein from melan-a cells cultured 5 days in the presence or absence of 10nM MSH, in the presence or absence of 10nM agouti protein, or in the presence of both MSH and agouti protein.

7

Figure 13 shows the Northern blot analysis of RNA levels of tyrosinase, TRP 1, TRP 2, MSH-R and GAPDH from melan-a cells cultured 5 days in the presence or absence of 10nM MSH, in the presence or absence of 10nM agouti protein, or in the presence of both MSH and agouti protein.

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Detailed Description Of The Invention

The present invention is a physiological extrinsic inhibitor of melanogenesis in mammals. An inhibitor of melanogenesis of the present invention is a

10 protein and peptides thereof that inhibit the production of melanin. A preferred inhibitor of melanogenesis of the present invention is agouti signaling protein and biologically active peptides thereof. Thus, the invention is a method of treating a hyperpigmentary condition in a subject, comprising administering to the subject the agouti signaling protein or a biologically active peptide thereof. The agouti signaling

15 protein and peptides thereof are useful for cosmetic purposes and for clinical application in the prevention or treatment of various hyperpigmentary conditions and diseases. Such conditions or diseases include but are not limited to melasma photoaging spots, solar keratosis, and post-inflammatory hyperpigmentation such as occurs at sites of wound healing.

20

Polypeptide and peptide are terms used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The polypeptide and peptide may be a synthetic peptide or polypeptide, recombinant peptide

25 or recombinant polypeptide or a peptide or polypeptide derived from enzymatic cleavage of the naturally occurring full length protein.

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Synthetic peptide refers to a chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

5   As used herein, the phrase "agouti signaling peptide" refers to a polypeptide or peptide having an amino acid residue sequence that comprises an amino acid residue sequence that corresponds, and preferably is identical, to a portion of the murine agouti signaling protein or the human homolog. The amino acid residue sequence of the mature murine agouti signaling protein is disclosed in Miller et al Gene

10   & Development 7:454-467, 1993 and is listed as SEQ ID NO: 1. The amino acid sequence of the mature human homolog to the agouti signaling protein is disclosed in Wilson, B.D. et al Human Mol. Genetics, 4(2):223-230, 1995 and is listed as SEQ ID No: 2. A peptide of the present invention has the capacity to inhibit the production of melanin.

15

Agouti Signal Protein (131 residues for murine)

H₂-MET-ASP-VAL-THR-ARG-LEU-LEU-LEU-ALA-THR-LEU-VAL-SER-PHE- (mouse)
(SEQ ID NO:1)

MET–ASP–VAL–THR–ARG–LEU–LEU–LEU–ALA–THR–LEU–LEU–GLY–PHE–
(human) (SEQ ID NO:2)
     signal sequence -   • •

LEU-CYS-PHE-PHE-THR-VAL-HIS-SER-HIS-LEU-ALA-LEU-GLU-GLU-
LEU-CYS-PHE-PHE-THR-ALA-ASN-SER-HIS-LEU-PRO-PRO-GLU-GLU-
          • • | • •

THR-LEU-GLY-ASP-ASP-ARG-SER-LEU-ARG-SER-ASP-SER-SER-MET-
LYS-LEU-ARG-ASP-ASP-ARG-SER-LEU-ARG-SER-ASP-SER-SER-VAL-
  •   •   - basic region -   •

ASN-SER-LEU-ASP-PHE-SER-SER-VAL-SER-ILE-VAL-ALA-LEU-ASN-
ASN-LEU-LEU-ASP-VAL-PRO-SER-VAL-SER-ILE-VAL-ALA-LEU-ASN-
     •   • •

LYS-LYS-SER-LYS-LYS-ILE-SER-ARG-LYS-GLU-ALA-GLU-LYS-ARG-

SUBSTITUTE SHEET (RULE 26)

LYS-LYS-SER-LYS-GLN-ILE-GLY-ARG-LYS-ALA-ALA-GLU-LYS-LYS-
    •   •   •   •

LYS-ARG-SER-SER-LYS-LYS-LYS-ALA-SER-MET-LYS-LYS-VAL-ALA-
----ARG-SER-SER-LYS-LYS-GLU-ALA-SER-MET-LYS-LYS-VAL-VAL-
    •   •   •

ARG-PRO-PRO-PRO-PRO-SER-PRO-CYS-VAL-ALA-THR-ARG-ASP-SER-
ARG-PRO-PRO-PRO-PRO-SER-PRO-CYS-VAL-ALA-THR-ASN-ASP-SER-
            |---   cysteine rich motif-- -   •

CYS-LYS-PRO-PRO-ALA-PRO-ALA-CYS-CYS-ASP-PRO-CYS-ALA-SER-
CYS-LYS-PRO-PRO-ALA-PRO-ALA-CYS-CYS-ASP-PRO-CYS-ALA-SER-

CYS-GLN-CYS-ARG-PHE-PHE-GLY-SER-ALA-CYS-THR-CYS-ARG-VAL-
CYS-GLN-CYS-ARG-PHE-PHE-GLY-ARG-ALA-CYS-SER-CYS-ARG-VAL-
                •   •

LEU-ASN-PRO-ASN-CYS-CO_2H
LEU-SER-LEU-ASN-CYS-CO_2H
    •   •

*Difference

An agouti signaling peptide of the present invention is derived from a basic region or portion thereof, a cysteine rich region or portion thereof or a combination of a basic region and a cysteine rich region.

5   An agouti signaling peptide of the present invention is preferably no more than about 131 amino acid residues in length for reasons of ease of synthesis and ability to direct the inhibition. Thus, it is more preferred that an agouti signaling peptide be no more than about 109 amino acid residues, still more preferably no more than about 50 residues, and most preferably less than 20 amino acid residues in length. In one
10   embodiment, an agouti signaling peptide of the present invention has about 5 to about 10 amino acid residues and has the ability to inhibit melanin production.

          The invention is a purified biologically active peptide of the agouti signaling protein which has the following characteristics: (a) the peptide has depigmenting activity
15   due to its ability to inhibit the production of at least one melanin; (b) the peptide has at least one region selected from the group consisting of: (i) at least one basic region

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derived from the full length agouti signaling protein or a portion thereof; and (ii) a
cysteine rich region derived from the full length agouti signaling protein or a portion
thereof; (c) the peptide has a minimum length of at least about 5 amino acid residues but
has a maximum length selected from the group consisting of: (i) no more than about 131
5   amino acid residues; (ii) no more than about 109 amino acid residues; (iii) no more than
about 50 amino acid residues; (iv) no more than about 20 amino acid residues; and (v)
no more than about 10 amino acid residues; and wherein said agouti signaling protein is
optionally coupled to a molecule which would facilitate its transport into cells. In a
specific embodiment, the agouti signaling protein having these characteristics and a
10   maximum length of no more than about 131 amino acid residues is provided. In a
further specific embodiment, the agouti signaling protein has a maximum length of no
more than about 109 amino acid residues.

In one embodiment, an agouti signaling peptide of the present invention has a
15   length of no more than about 109 amino acid residues and includes an amino acid
residue sequence or biologically active portion thereof represented by the formula:
His-Leu-Ala-Leu-Glu-Glu-Thr-Leu-
Gly-Asp-Asp-Arg-Ser-Leu-Arg-Ser-
Asp-Ser-Ser-Met-Asn-Ser-Leu-Asp-
20   Phe-Ser-Ser-Val-Ser-Ile-Val-Ala-
Leu-Asn-Lys-Lys-Ser-Lys-Lys-Ile-
Ser-Arg-Lys-Glu-Ala-Glu-Lys-Arg-
Lys-Arg-Ser-Ser-Lys-Lys-Lys-Ala-
Ser-Met-Lys-Lys-Val-Ala-Arg-Pro (SEQ ID NO:3).
25

In another embodiment an agouti signaling peptide of the present invention has a
length of no more than about 109 amino acid residues and includes an amino acid
residue sequence or biologically active portion thereof represented by the formula:
His-Leu-Pro-Pro-Glu-Glu-Lys-

Leu-Arg-Asp-Asp-Arg-Ser-Leu-
Arg-Ser-Asp-Ser-Ser-Val-Asn-
Leu-Leu-Asp-Val-Pro-Ser-Val-
Ser-Ile-Val-Ala-Leu-Asn-Lys-
5   Lys-Ser-Lys-Gln-Ile-Gly-Arg-
Lys-Ala-Ala-Glu-Lys-Lys-Arg-
Ser-Ser-Lys-Lys-Glu-Ala-Ser-
Met-Lys-Lys-Val-Val-Arg-Pro (SEQ ID NO:4)

       In another embodiment an agouti peptide of the present invention has a length of
10   no more than about 109 amino acids, preferably less than about 50 amino acids, and
includes an amino acid sequence or portion thereof represented by the formula:
Pro-Pro-Pro-Ser-Pro-Cys-Val-Ala-Thr-Arg-
Asp-Ser-Cys-Lys-Pro-Pro-Ala-Pro-Ala-
Cys-Cys-Asp-Pro-Cys-Ala-Ser-Cys-
15   Gln-Cys-Arg-Phe-Phe-Gly-Ser-Ala-
Cys-Thr-Cys-Arg-Val-Leu-Asn-Pro-
Asn-Cys (SEQ ID NO:5)

       In another embodiment an agouti peptide of the present invention has a length of
20   no more than about 109 amino acids, preferably less than about 50 amino acids and
includes an amino acid sequence or portion thereof represented by the formula:
Pro-Pro-Pro-Ser-Pro-Cys-Val-Ala-Thr-
Asn-Asp-Ser-Cys-Lys-Pro-Pro-Ala-
Pro-Ala-Cys-Cys-Asp-Pro-Cys-Ala-
25   Ser-Cys-Gln-Cys-Arg-Phe-Phe-Gly-Arg-
Ala-Cys-Ser-Cys-Arg-Val-Leu-Ser-
Leu-Asn-Cys (SEQ ID NO:6)

       In one embodiment, a preferred agouti signaling peptide includes at least one
30   amino acid residue sequence or portion thereof represented by SEQ ID NO:3 or SEQ

12

ID NO:4 and includes at least one amino acid residue sequence or portion thereof represented by SEQ ID NO:5 or SEQ ID NO:6 or combinations thereof.

Other exemplary peptides of the invention include but are not limited to peptides
5   having an amino acid residue sequence selected from the group consisting of:
     His-Leu-Ala-Leu-Glu-Glu-Thr-Leu-Gly-Asp (SEQ ID NO:7);
     His-Leu-Pro-Pro-Glu-Glu-Lys-Leu-Arg-Asp (SEQ ID NO:8);
     Asp-Arg-Ser-Leu-Arg-Ser-Asp-Ser-Ser-Met (SEQ ID NO:9);
     Asp-Arg-Ser-Leu-Arg-Ser-Asp-Ser-Ser-Val (SEQ ID NO:10);
10   Asn-Ser-Leu-Asp-Phe-Ser-Ser-Val-Ile-Val (SEQ ID NO:11);
     Asn-Leu-Leu-Asp-Val-Pro-Ser-Val-Ile-Val (SEQ ID NO:12);
     Ala-Leu-Asn-Lys-Lys-Ser-Lys-Lys-Ile-Ser (SEQ ID NO:13);
     Ala-Leu-Asn-Lys-Lys-Ser-Lys-Gln-Ile-Gly (SEQ ID NO:14);
     Arg-Lys-Glu-Ala-Glu-Lys-Arg-Lys-Arg-Ser (SEQ ID NO:15);
15   Arg-Lys-Ala-Ala-Glu-Lys-Lys——Arg-Ser (SEQ ID NO:16);
     Ser-Lys-Lys-Lys-Ala-Ser-Met-Lys-Lys-Val (SEQ ID NO:17);
     Ser-Lys-Lys-Glu-Ala-Ser-Met-Lys-Lys-Val (SEQ ID NO:18);
     Ala-Arg-Pro-Pro-Pro-Pro-Ser-Pro-Cys-Val (SEQ ID NO:19);
     Val-Arg-Pro-Pro-Pro-Pro-Ser-Pro-Cys-Val (SEQ ID NO:20);
20   Ala-Thr-Arg-Asp-Ser-Cys-Lys-Pro-Pro-Ala (SEQ ID NO:21);
     Ala-Thr-Asn-Asp-Ser-Cys-Lys-Pro-Pro-Ala (SEQ ID NO:22);
     Pro-Ala-Cys-Cys-Asp-Pro-Cys-Ala-Ser-Cys (SEQ ID NO:23);
     Pro-Ala-Cys-Cys-Asp-Pro-Cys-Ala-Ser-Cys (SEQ ID NO:24);
     Gln-Cys-Arg-Phe-Phe-Gly-Ser-Ala-Cys-Thr (SEQ ID NO:25);
25   Gln-Cys-Arg-Phe-Phe-Gly-Arg-Ala-Cys-Ser (SEQ ID NO:26);
     Cys-Arg-Val-Leu-Asn-Pro-Asn-Cys (SEQ ID NO:27);
     Cys-Arg-Val-Leu-Ser-Leu-Asn-Cys (SEQ ID NO:28);

13

repeating sequences and combinations of one or more of the sequences thereof having the ability to inhibit the production of at least one melanin. The peptides may be screened for inhibitory activity using the methods described herein.

5   Due to the three dimensional structure of a native folded agouti signaling protein, multiple regions of agouti signaling protein may be involved in inhibiting melanin production. Thus, in another embodiment, the invention contemplates agouti signaling peptide compositions that comprise one or more of the different agouti signaling peptides described above, admixed in combinations to provide simultaneous

10   inhibition of melanin. Similarly, mosaic polypeptides comprising two or more of the agouti signaling peptides, linked by other than the normal intervening amino acid sequences is also contemplated.

It should be understood that a subject peptide need not be identical to the amino

15   acid residue sequence of SEQ ID NO: 1 or 2 so long as it includes the required sequence and is able to inhibit the production of at least one melanin as described herein.

A subject polypeptide includes any analog, fragment or chemical derivative of a polypeptide whose amino acid residue sequence is shown herein so long as the

20   polypeptide is capable of inhibiting the production of at least one melanin. Therefore, a present polypeptide can be subject to various changes, substitutions, insertions, and deletions where such changes provide for certain advantages in its use.

The term "analog" includes any peptide having an amino acid residue sequence substantially identical to a sequence specifically shown herein in which one or more

25   residues have been conservatively substituted with a functionally similar residue and which displays the ability to inhibit the production of melanin as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and

14

lysine; between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically
5   derivatized residue in place of a non-derivatized residue provided that such peptide displays the requisite inhibitory activity.

"Chemical derivative" refers to a subject peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized
10   molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-
15   alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methlyhistidine may be substituted for histidine;
20   homoserine may be substituted for serine; and ornithine may be substituted for lysine. Peptides of the present invention also include any peptide having one or more additions and/or deletions or residues relative to the sequence of a peptide whose sequence is shown herein, so long as the requisite activity is maintained.

25   The term "fragment" refers to any subject peptide having an amino acid residue sequence shorter than that of a polypeptide whose amino acid residue sequence is shown herein.

SUBSTITUTE SHEET (RULE 26)

When a peptide of the present invention has a sequence that is not identical to the sequence of agouti signaling protein, it is typically because one or more conservative or non-conservative substitutions have been made, usually no more than about 30 percent, preferably no more than about 20 percent, and more preferably no more than

5   about 10 percent of the amino acid residues are substituted. Additional residues may also be added at either terminus for the purpose of providing a "linker" by which the peptides of this invention can be conveniently affixed a carrier.

Carrier molecules may be used to provide stability to the peptide of the present

10   invention and/or may be used to target the peptide to a particular site or a particular target cell. For example, the carrier may be a melanocyte receptor or melanocyte specific ligand that targets the agouti peptide to melanocytes. In another embodiment, the carrier is an auxiliary peptide which can facilitate delivery of the peptide to the target cells. An example of such a peptide is Penetratin sold by Appligene, a division of Oncor.

15   The peptide is patented and has 16 amino acids. Other carrier molecules comprising peptides, sugars, lipids, etc. can be joined to the agouti signaling protein or peptides thereof to facilitate delivery to a target cell, improve stability or provide other useful functions.

20   In one embodiment a more stable derivative of the agouti signaling peptide is synthesized by protocols as described by Hadley, M.E. et al Endocrine Res. 11(3-4)157-170, 1985.

Amino acid residue linkers may be used to link the agouti peptide to the carrier

25   molecule and are usually at least one residue and can be 40 or more residues, more often 1 to 10 residues. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic and aspartic acid, or the like. In addition, a subject peptide can differ, unless otherwise specified, from the natural sequence of agouti signaling protein by the sequence being modified by terminal-NH_2 acylation, e.g., acetylation, or thioglycolic

acid amidation, by terminal-carboxylamidation, e.g., with ammonia, methylamine, and the like.

5   Any peptide of the present invention may be used in the form of a pharmaceutically acceptable salt. Suitable acids which are capable of forming salts with the peptides of the present invention include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid,

10   naphthalene sulfonic acid, sulfanilic acid or the like.

       Suitable bases capable of forming salts with the peptides of the present invention include inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium · hydroxide and the like; and organic bases such as mono-, di- and tri-alkyl and aryl

15   amines (e.g. triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanolamines (e.g. ethanolamine, diethanolamine and the like).

       An agouti signaling peptide of the present invention, can be synthesized by any

20   of the techniques that are known to those skilled in the polypeptide art, including recombinant DNA techniques. Methods for construction, expression and purification of recombinant proteins are detailed in: Current Protocols in Molecular Biology, Vols. 1-3, Eds. Ausubel, F.M. et al, John Wiley & Son, Inc., 1995. For producing short peptides, synthetic chemistry techniques, such as a solid-phase Merrifield-type synthesis, are

25   preferred for reasons of purity, freedom from undesired side products, ease of production and the like. An excellent summary of the many techniques available can be found in J.M. Steward and J.D. Young, "Solid Phase Peptide Synthesis", W.H. Freeman Co., San Francisco, 1969; M. Bodanszky et al, "Peptide Synthesis", John Wiley & Sons, Second Edition, 1976 and J. Meienhofer, "Hormonal Proteins and Peptides", Vol. 2, p.

46, Academic Press (New York), 1983 for solid phase peptide synthesis, and E. Schroder and K. Kubke, "The Peptides", Vol. 1, Academic Press (New York), 1965 for classical solution synthesis, each of which is incorporated herein by reference. Appropriate protective groups usable in such synthesis are described in the above tests and in J.F.W. McOmie, "Protective Groups in Organic Chemistry", Plenum Press, New York, 1973, which is incorporated herein by reference.

5

The invention is a purified antibody that specifically binds a peptide of the invention. Particularly, the peptide can be a polyclonal antibody that specifically binds to a carboxy-terminal peptide having the sequence CGLGENSPLLSGQQV (SEQ ID NO:29). In a further embodiment a purified monoclonal antibody that specifically binds the peptide having the sequence of SEQ ID NO:29. Purified polyclonal and monoclonal antibodies to other novel peptides of the invention are contemplated.

10

The present invention is a method of inhibiting at least one melanogenic enzyme, preferably inhibiting more than one melanogenic enzyme. Because the skin pigmentation genes cloned thus far show high levels of sequence similarity and have the same functions between mice and humans, the results of the experiments on mice are clearly correlated to expected results in humans. Melanogenic enzymes which may be inhibited or down-regulated include but are not limited to tyrosinase, TRP1 and human homolog thereof, TRP2 and human homolog thereof and the like.

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In one embodiment of the invention the method of inhibiting or down-regulating a melanogenic enzyme results in inhibition of tyrosinase, TRP1 and TRP2. Thus a method of down-regulating in a subject a melanogenic enzyme involved in melanin synthesis, comprising administering to the subject an amount of the agouti signaling protein or a biologically active peptide thereof to down-regulate the melanogenic enzyme is provided. In another embodiment of the invention the method results in inhibition of the synthesis of at least one melanin. Such melanins which may be inhibited

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18

by the present invention include but are not limited to eumelanin and pheomelanin. In a preferred embodiment the method results in the inhibition of production of at least eumelanin.

5   The method is the administration of agouti signal protein or one or more biologically active peptides thereof to a mammal in an amount sufficient to inhibit or decrease the production or synthesis of at least one melanin, for example, eumelanin and pheomelanin.

10   The present method is particularly useful in the treatment of hyperpigmentation conditions or diseases which include but are not limited to melasma, photoaging spots, solar keratosis, post-inflammatory hyperpigmentation, and the like.

        Agouti signaling protein and biologically functional peptides thereof are useful in
15   methods of inhibiting melanin production at a site of hyperpigmentation in a mammal, preferably a human. Agouti signaling protein or biologically functional peptide thereof administered at such sites prevents or inhibits the formation of melanin at the site. Thus, the invention is a method of reducing melanin synthesis in a subject, comprising administering to a subject in need of such reduction an amount of the agouti signaling
20   protein or a biologically active peptide thereof to reduce melanin synthesis.

        In the method of treatment, the administration of agouti signaling protein or biologically functional peptides thereof may be provided for either prophylactic or therapeutic use. When provided prophylactically, the agouti signaling protein or
25   biologically functional peptides thereof is provided in advance of any overproduction of melanin at a site. The prophylactic administration of the protein or peptides of the present invention serves to prevent or inhibit any melanin overproduction at the site. When provided therapeutically, the protein or peptide is provided at (or after) the onset of melanin production at a site. Thus, the protein or peptide may be provided either

prior to the anticipated melanogenesis at the site or after melanogenesis has begun at a site.

The term "unit dose" as it is used herein refers to physically discrete units
5 suitable as unitary dosages for mammals, each unit containing a predetermined quantity of agouti signaling protein, peptides and derivatives thereof calculated to produce the desired inhibitory effect.

The inoculum is typically prepared as a solution in tolerable (acceptable) diluent
10 such as saline, phosphate buffered saline or other physiologically tolerable diluent and the like to form an aqueous pharmaceutical composition. In addition, the protein, peptides and derivatives thereof may be formulated in solid form and lyophilized form and redissolved or suspended prior to use.

15 The composition may optionally contain other therapeutics. Of particular interest are therapeutics useful in the prevention or treatment of damaged skin. For example, the composition may also comprise estrogen or derivatives thereof, tretinoin or Vitamin A derivatives, UV-A and/or UV-B sun-blocking agents, antibiotics, acne treatment agents, and the like. For example, the composition may comprise an effective
20 concentration of agouti signaling peptide in combination with an effective concentration of tretinoin formulated as a cream. The concentration of tretinoin may be from about 0.025% to about 0.1% tretinoin.

The agouti signaling protein or peptides may also be formulated into a cosmetic
25 composition.

The route of administration may be subcutaneous (S.C.), intradermal (I.D.), topical and the like so as to be directed to melanocytes. One embodiment of the method of treatment the protein, peptide or derivatives thereof are administered topically. The

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peptides of the present invention may be modified by the addition of a carrier group that facilitates their penetration through the skin. Such groups include but are not limited to lipophilic groups and the like. Examples of lipophilic groups are fatty acids or fatty alcohols in addition to long chain hydrocarbyl groups. Other formulations for enhancing

5   epidermal, dermal and transdermal penetration of topically applied pharmacologically active agents is disclosed in U.S. Patent Nos. 5,326,566, 5,409,917 and 5,260,292. Alternatively, the peptides of the present invention may be formulated into liposomes to facilitate their entry using topical administration. For topical administration, the protein, peptide or derivatives thereof is formulated into ointments, salves, gels, dermal patches

10   or creams, as is generally known in the art.

In providing a mammal, preferably a human, with the agouti signaling protein or peptide, the dosage of administration of the protein, peptides or derivatives thereof will vary depending upon such factors as the mammal's age, weight, height, sex, general

15   medical condition, previous medical condition, route of administration, formulation and state of progression of the hyperpigmentation.

In general, it is desirable to provide the recipient with a dosage of agouti signaling protein or peptide of at least about picomolar concentrations, preferably at

20   least about nanomolar concentrations, although a lower or higher dose may be administered. In the case of stable derivatives with long half-lives, lower doses may be effective such as, but not limited to, a picomolar range. The dose provides an effective tissue level of agouti signaling protein or peptides thereof for inhibiting melanogenesis at a site. The dose is administered at least once. Multiple administration over a period of

25   hours, days or weeks may be preferable. It may also be preferable to administer the protein or peptide at least once/week and even more frequent administrations. Subsequent doses may be administered as indicated.

In one embodiment the agouti signaling protein or peptides thereof in provided topically in the form of a lotion that is applied to the affected skin of the hands and face on a daily basis.

5   The methods described herein are useful in screening analogs, derivatives and fragments of the agouti signaling protein for those useful in inhibiting melanogenesis. In the method, an amount of an agouti protein or peptide is added to cultured primary melanocytes or melanocyte cell lines. At various time intervals, samples are removed and the amount of melanocyte enzyme determined. Thus, the invention is a method of

10   screening for inhibitors of melanin synthesis, comprising: a) contacting a melanocyte culture with an amount of a putative inhibitor of melanin synthesis; b) determining the amount of melanocyte enzyme present in the melanocyte culture from step a); c) comparing the amount of melanocyte enzyme determined in step b) to the amount of melanocyte enzyme in an uncontacted melanocyte culture; and d) correlating a decrease

15   in at least one melanocyte enzyme with an inhibitor of melanin synthesis. A decrease in at least one melanocyte enzyme compared to a control is indicative of an inhibitory agouti protein or peptide.

There are genetic conditions that result in over-expression of agouti signaling protein. One example is the lethal yellow mutation in mice. Other conditions or

20   diseases such as melasma photoaging spots, solar keratosis, and post-inflammatory hyperpigmentation such as occurs at sites of wound healing are related to the over-expression of a melanin. Thus another aspect of the invention is the modulation of expression of endogenous agouti signaling protein in a mammal. Expression of endogenous agouti signaling protein may be inhibited at the protein level by agents such

25   as anti-agouti signaling protein antibody, FAb fragments and the like or by chemical agents that bind to the agouti protein thus preventing its function.

At the transcription or translation level, anti-sense oligonucleotides may be used to prevent the expression of the agouti signaling protein. Such anti-sense

22

oligonucleotides are formulated based on the nucleic acid sequence encoding the protein
of SEQ ID NO:1 or SEQ ID NO:2 and can be made by methods known in the art. Thus,
the invention includes a method of altering melanin synthesis in a subject, comprising
administering to the subject an amount of a nucleic acid that hybridizes to a nucleic acid
5   encoding an agouti signaling protein, whereby the agouti encoding nucleic acid is not
transcribed or translated and melanin synthesis is altered. This treatment results in
enhanced eumelanogenesis, which can be beneficial in treating vitiligo, leucoderma,
some forms of albinism and hair graying.

10   This technique can be antisense RNA therapy. The general protocol is to
identify the gene that causes disease in humans. The agouti signaling protein-encoding
gene is such a gene. Next, the gene is cloned (Bultman et al., 1992 and Miller et al.,
1993), but in a reverse orientation and with an powerful inducible exogenous promoter.
This construct is then integrated into a cell that expresses the gene, the promoter is
15   activated by an inducing compound, and "antisense" RNA in produced. This RNA is
capable of binding to "sense" RNA (e.g., mRNA) that is produced in the disease state.
By blocking the sense RNA, translation is prevented, and the product's effect in causing
disease is halted.

20   To get the antisense molecules into the target cells a carrier molecule will be
covalently linked to the antisense molecule. This carrier moiety will take the form of a
necessary/required biochemical compound for the agouti signaling protein-expressing
cells. The cell will be tricked into accepting the carrier molecule and, thus, the entire
antisense molecule. Binding kinetics will take over form this point. In the case of
25   antisense DNAs, much smaller numbers of anti-ASP molecules should be required to
shut down transcription, perhaps translation, and ultimately impact melanin production.

It is understood that the examples and embodiments described herein are for
illustrative purposes only and that various modifications or changes in light thereof will

23

be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

All references and patents referred to are incorporated herein by reference.

5

Example 1

Materials and Methods

Animals

10   C3H/HeJ agouti mice (A/A: B/B: C/C) and C57BL/6J (C3H/HeJ F12 mice (A/a: B/B: C/C) were purchased from Charles River (Kingston, NY). C57BL/6J nonagouti black mice (a/a: B/B: C/C) and C57BL/6J lethal yellow mice (A/a: B/B: C/C) were obtained from Jackson Laboratory (Bar Harbor, ME). Lethal yellow mice were maintained by matings between heterozygous lethal yellow (A/a) male and black (a/a)

15   female mice. Sibling nonagouti (a/a) and heterozygous (a/a) mice and female C57BL/6J (C3H/HeJ F1 (A/a) mice. All mice were housed in the animal facility of NCI/HIH.

Antibodies used

Antibodies used in this study were generated in rabbits against synthetic peptides

20   corresponding to the unique carboxyl sequence of the four melanogenic proteins studied; their specificities have been thoroughly detailed in the appropriate references. They are termed αPEP1 (which recognizes TRP1; Jimenez et al., 1991), αPEP7 (which recognizes tyrosinase; Jimenez et al., 1991), αPEP8 (which recognizes TRP2; Tsukamoto et al., 1992) and αPEP13 (which recognizes the silver protein; Kobayashi et

25   al, 1994a).

Sample preparation

Anesthetized mice were killed by cervical dislocation, and their dorsal skins were cleaned with 60% ethanol and dissected. Immediately after removing fat tissue, the

dorsal skins were homogenized in 5 volumes (v/v) of NP40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris HCl, pH 7.2 1 μg ml aprotinin and 100 μM phenylmethylsulfonyl fluoride) on ice using a Potter-Elvehjem glass homogenizer, or they were frozen and kept at -70°C until use. Following centrifugation at 1,000 g for 10

5   minutes at 4°C, the supernatant was subsequently centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was filtered through a 0.45 μm pore filter unit (Millex, Millipore Co., Bedford, MA) and the soluble skin extracts were used for menalogenic assays and western immunoblotting analyses, as detailed below. In some cases where noted, hair bulbs were collected from dorsal skins using razor blades.

10

Organ culture

This technique was carried out as previously described (Imokawa et al., 1988). Briefly, dorsal skins were excised from 6- or 10-day-old mice, cleaned to remove the fat tissue and to expose the hair bulbs, and were then cut into pieces (~1mm x 2mm) using

15   razor blades. The pieces were sterilized in Hanks' balanced salt solution containing 400 i.u./ml penicillin and 400 μg/ml streptomycin for 20 minutes, and then placed on sheets of lens paper over stainless steel grids in organ culture dishes (Falcon 3037, Lincoln Park, NJ). A 750 μl sample of Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 100 i.u./ml penicillin and 100 μg/ml streptomycin, were added to the

20   inner plates of the dishes in order to culture the skin tissue at the liquid/air (5% CO_2) interface at 37°C.

Cells and culture conditions

The melan-a melanocyte cell line cultured from genetically defined C56Bl mice (a/a

25   B/B C/C) was a kind gift from Dr. Dorothy Bennett, London, United Kingdom. The cells were grown as initially reported (Bennett et al., 1987).

25

Metabolic labeling and immunoprecipitation

These techniques were performed as previously reported (Jimenez et al., 1989, 1991; Tsukamoto et al., 1992; Aroca et al., 1993; Kobayashi et al., 1994a). Briefly, dorsal skin fragments in organ culture were preincubated for 1 hour in methionine-free medium

5 containing dialyzed fetal bovine serum, and labeled for 4 or 6 hours with [^{35}S]methionine (0.4~1.0 mCi/ml) (Dupont-NEN, Boston, MA) in methionine-free medium. They were washed three times with ice-cold Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS-), containing excess unlabeled methionine, and the hair bulbs were collected from radiolabeled skin pieces using razor blades; these were homogenized and lysed overnight

10 at 4°C in NP40/SDS buffer, as detailed above. The lysates were then centrifuged for 30 minutes at 10,000 g, and the supernatants were then precleared with normal rabbit serum and GammaBind G Sepharose (Pharmacia/LKB, Piscataway, NJ). A sample containing 10^7 TCA-precipitable cpm of the precleared extracts was incubated with 5 μl of the antibodies noted in the Figure legends for 1 hour at 4°C, and then complexed

15 with 30 μl; GammaBind G Sepharose for 30 minutes at 4°C. The immune complexes were washed 5 times with NP40/SDS buffer, then eluted in SDS sample buffer at 95°C for 5 minutes and analyzed by SDS-gel electrophoresis (Laemmli, 1970), followed by fluorography.

20 ### Western immunoblotting analysis

This technique was also performed as reported previously (Jimenez et al., 1991; Aroca et al., 1993; Kobayashi et al., 1994a). Briefly, proteins from NP40/SDS-solubilized melan-a cells or dorsal skins were separated on 7.5% SDS gels, then transferred to polyvinylidene difluoride membranes (Immunobilon-P, Millipore Corp.,

25 Bedford, MA) and incubated with primary antibodies (1/1000 dilution) as noted in the Figure legends. Subsequent visualization of antibody binding was carried out with Enhanced ChemiLuminescence (Amersham Corp., Arlington Height, IL) according to the manufacturer's instructions.

26

Melanogenic assays

Assays for melanogenic catalytic activities as described below were carried out at pH 6.8, 37°C for 60 minutes. (1) Tyrosine hydroxylase activity was measured using the [^{3}H]tyrosine assay (Hearing and Ekel, 1976; Hearing, 1987). This method specifically
5   measures the tritiated water produced during the hydroxylation of tyrosine to DOPA. (2) DOPA oxidase activity was measured using incorporation of [3-^{14}C]DOPA into acid-insoluble melanin as detailed previously (Aroca et al., 1993). (3) DOPAchrome tautomerase activity was measured by HPLC as the disappearance of DOPAchrome substrate and the production of DHICA rather than DHI; data are converted to pmol
10   products by comparison with known standards. The HPLC assay was detailed previously (Palumbo et al., 1987; Tsukamoto et al., 1992). (4) DHI oxidase and DHICA oxidase activities were measured by HPLC as the disappearance of these substrates from reaction mixtures compared to controls for spontaneous auto-oxidation; data are converted to pmol by comparison with known standard (Aroca et al., 1993).
15   (5) Melanin production was measured using incorporation of [^{14}C]tyrosine into acid-insoluble melanin, also as detailed previously (Hearing and Ekel, 1976; Hearing, 1987); pmol melanin produced are calculated from the radioactive product.

Tyrosine and DOPA used as standards and reaction substrates in these assays
20   were obtained from Sigma Chemical Co. (St. Louis, MO); [L-3,5-^{3}H]tyrosine, [3-^{14}C]DOPA and [U-^{14}C]tyrosine were obtained from New England Nuclear (Boston, MA); DOPAchrome was prepared using the silver oxide method originally described by Korner and Pawelek (1980). DHI and DHICA were kindly provided by Dr. Giuseppe Prota (University of Naples, Naples) and Dr. Shosuke Ito (Fujita Health University,
25   Nagoya) and purchased commercially from Regis Chemical Co. (Morton Grove, IL).

RNA isolation and northern blotting

These techniques were performed as described previously (Aroca et al., 1993). Briefly, 2.0 µg of mRNA, isolated using a total RNA isolation kit and mRNA

27

purification kit (Promega, Madison, WI), were electrophoresed and then blotted to
Sure-Blot nylon membranes (Oncor, Gaithersburg, MD), hybridized with ^{32}P-labeled
probes, dehybridized and rehybridized with other probes again as necessary. TYRS-J,
the probe specific for tyrosinase, was kindly provided by Drs. H. Yamamoto and T.

5   Takeuchi, Sendai, Japan and is described in Yamamoto et al <u>Japan J. Genetics</u> Vol
62:271-274, 1987. pMT4, specific for TRP1, was provided by Dr. S. Shibahara,
Sendai, Japan and is described in Shibahara, S. et al <u>Nucleic Acid Res.</u> Vol 14:2413-
2427, 1986. TRP2a, specific for TRP2 was provided by Dr. I. Jackson, Edinburgh,
Scotland and is described in Jackson, I et al. <u>EMBO</u> Vol 11:527-535, 1992. gp100,

10   specific for silver, was provided by Dr. Y. Kawakami, Bethesda, MD and is described in
Kawakami et al <u>PNAS,</u> Vol. 91, 6458-6462, 1994.

Miscellaneous methods

    Protein concentrations were determined with the BCA assay kit (Pierce Chem Co.,
15   Rockford, IL) using bovine serum albumin as the standard. Quantitation of western
blots and autoradiographs was performed using ImageQuant imager and software.

<u>Example 2</u>

20   **Melanogenic protein expression in hair bulbs of newborn lethal yellow and
black mice.**

    The expression of melanogenic proteins during pheomelanogensis was studied by
comparing dorsal hair bulbs of 10 day newborn lethal yellow and black mice; these were
25   sibling offspring from the same litter. Ten days newborn lethal yellow mice produce
predominantly pheomelanin in their hair bulb although younger lethal yellow mice make
minor amounts of eumelanin and brownish pigment can occasionally be observed in their
hair bulbs.

28

The three distinct tyrosinase activities (i.e. tyrosine hydroxylase, DOPA oxidase and DHI oxidase), as well as the ability to produce melanin in extracts of hair bulbs from lethal yellow mice were about 20-25% the levels found in extracts of hairbulbs from the black sibling mice (Table 1). However, levels of DOPAchrome tautomerase activity (i.e. TRP2) and DHICA oxidase activity (i.e. TRP1) in these extracts of pheomelanogenic hair bulbs were at background levels. The relatively higher specific activities of these catalytic functions measured in pure population of melan-a melanocytes are shown for comparison.

Table 1. Melanogenic activities in hair bulbs from newborn lethal yellow and black mice

Sample	Tyrosine hydroxylase	DOPA oxidase	DOPAchrome tautomerase	DHI oxidase	DHICA oxidase	Melanin formation
Lethal yellow	12±4 (n=5)	150±57 (n=5)	0.3±0.6 (n=6)	23±7 (n=5)	-10±17 (n=3)	3±1 (n=5)
Black	46±21 (n=5)	538±270 (n=5)	93±72 (n=6)	131±83 (n=5)	21±31 (n=3)	10±6 (n=5)
Melan-a	110±13 (n=3)	1039±13 (n=3)	114±60 (n=3)	432±140 (n=7)	319±111 (n=7)	33±6 (n=7)

Hair bulbs from 10 day newborn lethal yellow and black sibling mice (or melan-a cells in culture) were solubilized and assayed for melanogenic activities, as detailed in Materials and Methods. Data are reported as means ± s.e.m. in pmol/mg protein per hour; n = number of independent experiments.

By western immunoblotting analysis, the patterns of expression of tyrosinase, TRP1 and TRP2 were consistent with the results of melanogenic assays (Fig. 2). Extracts of hair bulbs derived from newborn black mice had all four melanosomal proteins (tyrosinase, TRP1, TRP2 and the silver protein) with the same molecular mass
5   as found for the melan-a murine melanocyte line used as a positive control. However, a significantly lesser amount of tyrosinase was detected in hair bulbs derived from newborn lethal yellow mice, and TRP1, TRP2 and the silver protein were undetectable in those pheomelanic hair bulbs. Metabolic labeling with [35S]methionine followed by immunoprecipitation analysis of dorsal hair bulbs grown in organ culture showed similar
10   patterns for synthesis of melanogenic proteins by lethal yellow and black melanocytes (data not shown). In hair bulbs derived from black mice, tyrosinase, TRP1 and TRP2, as well as the silver protein, were detectable and comparable in size to those found in the

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melan-a cells. However, in hair bulbs derived from lethal yellow mice, significantly
decreased tyrosinase synthesis was detected, and there was no significant expression of
TRP1, TRP2 or the silver protein.

Northern blot analysis confirmed that the pheomelanogenic hair bulbs of lethal
yellow mice produced only tyrosinase mRNA but little or no mRNA for TRP1, TRP2 or
the silver protein, although we could readily detect significant levels of mRNAs for all of
those melanogenic proteins in the eumelanogenic hair bulbs obtained from black or
agouti mice (Fig. 3).

Taken together, these results at the enzyme activity, translational and
transcriptional levels, show clearly that tyrosinase function is reduced in hair bulbs of
newborn lethal yellow mice while TRP1, TRP2 and the silver protein are not expressed
at all. Therefore, the functions of TRP1, TRP2 and the silver protein cannot be essential
for pheomelanogenesis.

Example 3

Expression of melanogenic proteins in regenerating hair bulbs of lethal yellow
and black adult mice

To examine whether similar patterns of melanogenic proteins were expressed in
regenerating hair bulbs of adult mice, anagen growth of hairs was induced by plucking
the dorsal telogen hairs of 2-month-old lethal yellow and black sibling mice.
Regenerating hairs on the dorsa could be observed 10 days after plucking in both types
of mice and hair growth in both genotypes occurred at identical rates. Pieces of the
regenerating dorsal skin were biopsied, homogenized, solubilized in NP40/SDS buffer
and then analyzed by western immunoblotting and melanogenic enzyme assays. The
expression of tyrosinase, TRP1 and TRP2 could be detected in regenerating hair bulbs
of black mice by western immunoblotting as early as 6 days after plucking (Fig. 4). The

levels of each of those proteins increased further at 8 and 10 days post-plucking and
remained constant at 12 days. However, expression of TRP1 and TRP2 could not be
detected in the regenerating pheomelanic hair bulbs of sibling lethal yellow mice at any
time up to 12 days post-plucking, although a reduced expression of tyrosinase
5   (compared to the black controls) was seen during this same time period. The silver
protein was not detected in any of these skin extracts by western blotting (not shown).
Assays for tyrosine hydroxylase and DOPAchrome tautomerase activities in those same
extracts showed results consistent with the western immunoblotting analysis (Table 2).

10   The levels of DHICA production in crude extracts of tissues is occasionally
significantly above background; this high background (~5-6 pmol in this experiment) is
presumably due to the presence of divalent metal cations in these crude samples, which
can catalyze this tautomerase reaction in the absence of active enzyme, as noted above.

Table 2. Melanogenic activities in skin extracts of newborn agouti and black mice

	Age (days)	Tyrosine hydroxylase	DOPA oxidase	DOPAchrome tautomerase	DHI oxidase	DHICA oxidase	Melanin formation
			Experiment 1				
agouti	5	40	33±1	3±0	39	9	0.5±0.0
agouti	11	3±0.	24±1	8±0	46	16	0.4±0.0
			Experiment 2				
agouti	5	6±0	63±1	5±0	31	2	1.1±0.1
black	5	10±0	114±2	9±0	37	13	2.4±0.1

Hair bulbs from dorsal skins of 5 days and 11 days newborn agouti mice (top) and from dorsal skins of
5 days newborn agouti and black sibling mice (bottom) were solubilized and assayed for melanogenic
activities, as detailed in Materials and Methods. Data are reported as means ± s.e.m. in pmol/mg
protein per hour.

Example 4

Patterns of melanogenic protein expression during the physiological switch
from pheo- to eumelanogenesis

C3H agouti mice have the typical agouti hair pattern, i.e. a yellow strips in two regions of the black background on each hair shaft. This pattern is generated by temporarily switching the type of melanin formed from eumelanin to pheomelanin and then back to eumelanin again during hair growth. The expression of melanogenic proteins in agouti hair bulbs before, during and after their pheomelanic phase was investigated to determine if down-regulation of TRP1, TRP2 and silver protein expression occurred physiologically as had been observed above in regenerating hair bulbs of lethal yellow mice. Western immunoblotting (Fig. 5 and quantitation of those blots) revealed a decrease (maximal at day 7) in the expression of TRP1 in follicular melanocytes of newborn agouti mice, exactly the time at which pheomelanin is produced predominantly. This can be compared with the eumelanogenic stage (at 9 and 11 days), at which levels of TRP1 increase; it can be seen that the amount of tyrosinase present in those tissues was relatively constant throughout this same time frame. Results consistent with these patterns of expression were obtained in melanogenic assays (Table 2, top), where levels of tyrosinase activities were comparable in extracts of 5 day and 11 day newborn agouti skins, whereas the catalytic activity of TRP1 (DHICA oxidase) at day 5 was significantly less (only about 50%) than that detectable at day 11. Although there was no significant decrease in the TRP2 band detected by western blot (Fig. 5), its catalytic function (DOPAchrome tautomerase) was decreased ~50% at day 5 compared to day 11.

The expression of these melanogenic proteins in hair bulbs of sibling 5 day and 11 day newborn heterozygous agouti (A/a) and homozygous nonagouti (a/a) black mice were also examined. Results similar to those described above were obtained by western immunoblotting analysis (Fig. 6A) and by melanogenic assays (Table 2, bottom); that is, expression of tyrosinase and TRP2 was relatively constant in both genotypes at day 5 and day 11, whereas expression of TRP1 was reduced ~50% during the pheomelanogenic phase (day 5) in the agouti hair bulbs. In the experiments shown in Figs. 5 and 6A, the silver protein was not detected by western blotting due to its low

concentration in these extracts. Therefore expression of the silver pattern using metabolic labeling and immunoprecipitation of organ culture of skin obtained from 5-day old agouti and black sibling mice (Fig. 6B) was examined. Those experiments demonstrated that, as found for TRP1, there was significantly less of the silver protein

5   synthesized in hair bulbs of 5-day-old agouti mice compared to hair bulbs from black mice.

Example 5

10   **Agouti protein suppresses expression and activity of tyrosinase and tyrosinase-related protein in murine melanocytes.**

To characterize the switch between eumelanogenesis and pheomelanogenesis, the responses of cultured melanocytes exposed to αMSH and/or agouti were

15   determined.

The melan-a melanocyte cell line was used as described in Example 1. This clonal line was derived from C57B1 nonagouti black mice. The growth medium was minimum essential medium containing 200nM TPA. B16F10 melanoma cells were also

20   used to compare the effect of agouti protein with melan-a. These cells were grown in Dulbecco's modified Eagle medium.

Recombinant agouti protein was purified from baculovirus system by Dr. Michael Ollmann in Dr. Gregory Barsh's laboratory by methods known in the art. 2 x

25   10^6 cells were seeded per 15cm diameter dish. The medium was changed everyday, and fresh agouti protein and/or αMSH added everyday usually at 10nM. To examine transcriptional regulation, total RNA was isolated and Northern blotting performed by methods described in Example 1. The A26 probe to the MSH-R was provided by Dr. R. Cone and is described in Mountjoy et al. Science 257:1248-1251, 1992. At the

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translational level, metabolic labeling was used in conjunction with immunoprecipitation as described in Example 1. Western blotting and enzyme assay was used to examine function.

5   First, it was determined whether the agouti protein had any effect on tyrosinase expression by Northern blotting. Melan-a cells were treated with 4 concentrations of agouti protein as indicated in Figure 7A. After 24 hours, the cells were harvested, total RNA isolated and hybridized with a probe for tyrosinase and detected by a phosphoimager. The activity of each band was measured by phosphoimager and the %

10   control was corrected using GAPDH as a standard. Though the incubation time was only 24 hours, there were significant dose dependent decreases in expression of tyrosinase at 10nM and 1nM of agouti protein. It should be noted that in vivo, pheomelanin is synthesized within 24 hours after agouti RNA is apparent. Longer · incubation times were also examined. 10nM of agouti protein was added for 1, 2 or 4

15   days. After 2 or 4 days incubation, the decreases in expression of tyrosinase were much more dramatic, resulting in complete inhibition by day 2 (Figure 7B).

       After 5 days incubation of melan-a cells with 10nM agouti protein, the color of pellet was changed from black to light brown. By ultrastructural study, after exposure

20   to agouti protein, the amount of melanosomes were decreased and pheomelanosome-like structures were found (Figure 8B).

       The effects of agouti protein on expression of TRP1, TRP2, and the MSH receptor were examined. After 24 hours of exposure to different concentrations of

25   agouti protein, the level of TRP1 and TRP2 RNAs were reduced, but not to the same extent as tyrosinase RNA, and there was no significant effect on the level of RNA for the MSH receptor (Figure 9).

34

To examine directly the interaction between agouti protein and MSH, and to determine whether agouti protein might affect MSH signaling via a change in receptor level, melan-a cells were exposed to 10nM agouti, 10nM MSH, or both for period of 5 days, and then RNA levels were measured for tyrosinase, TRP1, TRP2, and MSH

5   receptor itself. MSH alone produced almost a twofold increase in tyrosinase, a 50% increase in TRP2, and no significant increase in TRP1 or MSH receptor. Agouti alone produced nearly a fivefold decrease in levels of RNA for tyrosinase, TRP1 and TRP2, but again had no significant effect on the level of MSH receptor RNA (Figure 10). At these concentrations, simultaneous addition of both agouti and MSH produced a

10   response indistinguishable from MSH alone. These results confirm the ability of agouti protein to bring about physiologic changes in the absence of exogenous MSH, and suggest that the interaction of agouti and MSH is not mediated by an alteration in levels of MSH receptor itself.

15   However, steady state levels of RNA as measured by Northern hybridization do not reveal alterations in the expression of a gene product that might occur due to modulation of protein levels or protein function. To examine effects at the translational level, the melan-a cells were cultured under the same condition for 5 days, and then the cells were incubated with ^{35}S methionine for 4 hours. Then, an immunoprecipitation

20   analysis was performed. Results were observed in immunoprecipitation analysis of tyrosinase, TRP1 and TRP2 that were consistent with the Northern blotting (Figure 11).

To examine enzyme function, enzyme assays was performed on extracts of cells cultured under the same conditions for 5 days. The results of tyrosine hydroxylase,

25   DOPA oxidase, DOPAchrome tautomerase and melanin production assays were also consistent with the Northern blotting experiments. These activities were increased after exposure to MSH alone. However, they were decreased to background levels after exposure to agouti protein alone, and simultaneous addition of both agouti protein and MSH produced a response indistinguishable from MSH alone.

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Table 3
Enzyme Assay*

	Control	MSH only	Agouti Only	MSH + Agouti
tyrosine hydroxylase	5.9 ± 1.2	8.7 ± 2.8	-1.2 ± 0.2	10.2 ± 1.72
DOPA oxidase	17.3 ± 5.1	197.5 ± 17.4	-2.6 ± 3.4	202.4 ± 24.0
DOPA chome tautomerase	255.0	555.0	0.0	450.0
melanin production	2.1 ± 0.6	6.3 ± 0.5	0.22 ± 0.0	6.99 ± 0.53

*measures pmol/µg protein/hr

Melanogenic enzyme levels were also examined using Western blotting under the same conditions, and the results also were again consistent with Northern blotting (Figure 12). These results suggest that modulation of pigment cell enzyme activity by agouti protein or MSH in culture occurs primarily at the level of messenger RNA rather than translational efficiency or post-translational processing.

The effect of agouti protein and MSH observed for melan-a cells was confirmed using B16F10 murine melanoma cells. Similar effects were observed (Figure 13). However, note that the agouti protein did not reduce tyrosinase RNA below baseline, and that MSH receptor expression was elevated in these agouti-treated cells.

Agouti protein added to melan-a cells suppresses the expression of tyrosinase, TPR1 and TRP2, and causes phenomelanosomes to be produced in vitro. This assay

system is useful for helping to determine the molecular and biochemical events required for pigment type switching.

Example 6

Human Skin Equivalent Model

The behavior of human melanocytes in the skin equivalent model closely mimics their behavior in vivo. Therefore, the results obtained using the model are reasonably predictive of efficacy in humans. The skin equivalent model has been described by Archambault, M. et al, J. Invest. Dermatol. 104(5):859-867, 1995.

Briefly, human newborn fibroblasts are cultivated from explants of foreskin dermis in Dulbecco's modified Eagle's medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% bovine serum (Hyclone Labs, Logan, UT) and used at sixth passage.

Human melanocytes are cultured from dissociated newborn foreskin epidermis. Melanocytes at second passage are plated on a dermal equivalent or in tissue culture in tissue culture dishes (Becton-Dickinson, Lincoln Park, NJ).

Human keratinocyte cultures are established from newborn foreskin. Preconfluent second-passage keratinocyte cultures are trypsinized and seeded onto dermal equivalents.

Agouti signaling protein or peptides thereof are added to the cultures and the cells incubated for 1 to 5 days. The inhibitory effect on the human melanocytes is determined by Northern blot analysis, Western blot analysis and enzymes assays of tyrosinase, TRP1, TRP2 as described previously in Examples 1 and 5.

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Example 7

Modulation of expression and activity of melanosomal proteins in murine melanocytes by agouti signal protein in cell culture

Cells and Cell Culture Conditions

The melan-a melanocyte line (Bennett et al., 1987), derived from C57Bl nonagouti black mice (a/a B/B C/C), was a kind gift from Dr. Dorothy Bennett, London, United Kingdom. This clonal line was cultured in Dulbecco's minimal essential medium containing penicillin, streptomycin, sodium pyruvate, nonessential amino acids, 25 mM sodium bicarbonate, 5% fetal bovine serum, 200 nM phorbol-12-myristate-13-acetate and 100 µM 2-mercaptoethanol at pH 6.9, as described by Bennett et al. (1987). Cells were usually seeded at a density of 1.5 x 10^6 cells per 15 cm diameter dish. For 24 hour experiments, ASP and/or MSH were added when the cells were initially seeded. For 5 day experiments, ASP was added immediately, and MSH was added starting on the next day (4 days of treatment with MSH is routinely used for optimal stimulation (Aroca et al., 1993)). The concentrations of ASP and MSH used ranged from 0.01 to 10 nM, as detailed in the Figure and Table legends. To controls without ASP or MSH, similar volumes of storage buffer (20 mM PIPES, pH 6.8, 50 mM NaCl) were added. The cells were routinely cultured at 37°C in a humidified incubator with 5% CO_2, and all media were changed daily. Cells were harvested by brief treatment with trypsin/EDTA in the standard manner, and used for subculture, or were processed for RNA, protein or enzyme analysis, as detailed below.

Agouti Signal Protein

Recombinant mouse ASP was generated and purified using a baculovirus expression system as described in Ollmann et al. (in preparation). The preparation used for most of the experiments described here is ≥ 90% pure as estimated by analysis of

[5] silver-stained gels and inhibits activation of the MC1-R with a K_i of 2.2×10^{-10} M. At 37°, ASP retains activity for more than 48 hours in water or tissue culture media. The experiment described in Fig. 2 has also been repeated with an ASP preparation ≥ 99% pure with virtually identical results.

Electron Microscopy

[10] Cells were harvested, centrifuged for 5 minutes at 14,000 g at 4°C, and fixed for 2 hours at 23°C in 2% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3; the fixative was then removed and the samples were stored in phosphate-buffered saline containing 2% sucrose at 4°C. Samples were subsequently processed with graded alcohols and embedded in epoxy resin for electron microscopy in

[15] the usual manner. Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed with a Zeiss EM10 electron microscope, as previously detailed (Prota et al., 1995).

RNA Isolation and Northern Blotting

Total RNA was extracted from cells using an RNeasy total RNA isolation kit (QIAGEN, Catsworth, CA), following the manufacturer's instructions. 20 μg of total RNA was denatured, electrophoresed through 1.0% agarose gels, and transferred

5   overnight at 23°C to SureBlot nylon hybridization membranes (Oncor, Gaithersburg, MD) in the standard manner. Filters were prehybridized for 3 hours at 45°C with Hybrisol I solution (Oncor, 50% formamide, 10% dextran sulfate, 1% SDS and blocking reagent), and then hybridized with a ^{32}P-labeled probe. A 2.0-kb EcoRI fragment of TYRS-J, a 1.7-kb HindIII fragment of pMT4, a 1.75-kb EcoRI fragment of TRP2a, and

10   a 2.1-kb BamHI - SalI fragment of A26 were used to detect tyrosinase, TRP1, TRP2 and MC1-R mRNAs, respectively. TYRS-J was obtained from Drs. Hiroaki Yamamoto and Takuji Takeuchi, Sendai, Japan (Yamamoto et al., 1987); pMT4 was obtained from Dr. Shigeki Shibahara, Sendai, Japan (Shibahara et al., 1986); TRP2a was obtained from Dr. Ian Jackson, Edinburgh, Scotland (Jackson et al., 1992); A26 was obtained from Dr.

15   Roger Cone, Oregon (Mountjoy et al., 1992). A commercially available cDNA probe specific for glyceraldehyde-3-phospho-dehydrogenase (GAPDH) was used to standardize RNA loading on the blots. The cDNA probes were labeled using random primer extension and heated to 100°C for 10 minutes, then cooled on ice for 10 minutes prior to adding to the hybridization solution. Hybridization was performed with the

20   radiolabeled probes in Hybrisol I (3 x 10^7 cpm/10 ml) overnight at 45°C with gentle shaking. Following the incubation, the blots were washed for 10 minutes at 23°C with 2X SSC/10% SDS, then for 10 minutes with 0.2X SSC/0.5% SDS, and finally for 10 minutes with 0.1X SSC/0.1% SDS. The blots were then exposed in phosphoroimager cassettes at 23°C for 1 hour and the densities of the bands were scanned using

25   ImageQuant software. The % control for each probe was corrected for initial loading using comparison with the GAPDH standard. After each scan, residual probe was removed by incubating for 15 minutes at 100°C in 0.1X SSC/0.1% SDS in 10 mM Tris, pH 7.0. This was repeated as necessary until no remaining probe could be detected.

Metabolic Labeling and Immunoprecipitation

These techniques were performed as previously reported (Jiménez et al., 1989;

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1991; Tsukamoto et al., 1992; Aroca et al., 1993). Briefly, subconfluent cells growing in culture in 10 cm diameter dishes were preincubated for 1 hour at 37°C in prewarmed methionine-free medium, and then were radiolabeled for 6 hours with 0.4 mCi/flask of [³⁵S]methionine. The cells were then harvested and solubilized for 1 hour at 4°C with NP-40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1M Tris-HCl, pH 7.2, 100 µM phenylmethylsulfonylfluoride, 1 µg/ml aprotinin). The cell lysates were then centrifuged for 15 minutes at 14,000 g at 4°C, and the supernatants were pretreated overnight at 4°C with normal rabbit serum and GammaBind G Sepharose (Pharmacia/LKB, Piscataway, NJ) to reduce background. 5 X 10⁶ cpm of each preabsorbed supernatant was then incubated with 10 µl of the appropriate antibodies. The antibodies used were generated in rabbits against synthetic peptides corresponding to the unique carboxyl sequences of the three melanogenic proteins studied; they are termed αPEP1 (which recognizes TRP1, (Jiménez et al., 1991)), αPEP7 (which recognizes tyrosinase, (Jiménez et al., 1991)) and αPEP8 (which recognizes TRP2, (Tsukamoto et al., 1992)). Following incubation at 37°C for 1 hour, 50 µl of GammaBind G Sepharose was added to each tube and then further incubated with mixing for 20 minutes at 23°C. The GammaBind G Sepharose-antigen-antibody immune complexes were washed 4 times with NP40/SDS buffer at 23°C, and then denatured in SDS sample buffer by heating to 100°C for 3 minutes. Specifically bound proteins were then analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Western Immunoblotting Analysis

Cells in tissue culture were harvested and solubilized for 1 hour at 4°C with NP-40/SDS buffer, then centrifuged at 14,000 g for 15 minutes at 4°C, and the supernatants were recovered. Proteins from the NP-40/SDS solubilized cells were
5   separated on 7.5% SDS gels, and then transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P, Milipore Corp., Bedford, MA). Following blocking overnight at 23°C in 3% bovine serum albumin in TBS/Tween (0.1% Tween 20 in Tris buffered saline), the blots were incubated with primary antibodies (at 1/1000 dilution in TBS/Tween). Following four washes in TBS/Tween to reduce nonspecific binding,
10   subsequent visualization of specific antibodies bound was carried out with Enhanced ChemiLuminescence (Amersham Corp.,Arlington Heights, IL) according to the manufacturer's instructions.

Melanogenic Enzyme Assays

Melanogenic assays were routinely carried out on NP-40/SDS soluble extracts
15   (obtained as above) at pH 6.8, 37°C for 60 minutes. To determine tyrosinase activity, the tyrosine hydroxylase assay was used; this assay measures tritiated water produced during the hydroxylation of L-[3,5-^{3}H]tyrosine to 3,4-dihydroxyphenylalanine (DOPA) (Hearing and Ekel, 1976; Hearing, 1987). For DOPA oxidase activity, the production of acid insoluble melanin product from [3-^{14}C]DOPA was measured (Aroca et al., 1993).
20   To determine DOPAchrome tautomerase activity, the disappearance of DOPAchrome substrate and the production of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than the spontaneously derived product 5,6-dihydroxyindole (DHI) was measured by HPLC (Palumbo et al., 1987; Tsukamoto et al., 1992). To determine melanin production, the [U-^{14}C]tyrosine assay (Hearing and Ekel, 1976; Hearing, 1987) was
25   used. All radioactive precursors were obtained from DuPont-New England Nuclear. DOPAchrome was prepared using the silver oxide method (Körner and Pawelek, 1980), and DHI and DHICA used as standards were obtained from Pierce Chemical Co. (Rockford, IL) and from Prof. Shosuke Ito, Nagoya, Japan. Pmol product of the assays was calculated from radioactivity measured or by comparison with standard curves.

cAMP Assays

These assays were performed as previously detailed (Barker et al., 1995; Medrano et al., 1995). Briefly, cells were plated into 24 well plates at a density of 3 X 10^5 cells per well and allowed to grow with a single change of medium for 48 hours. The media were then removed from each well, and the cells were incubated for 40 minutes in the presence of αMSH, ASP and/or cholera toxin, following which the reactions were stopped with 1 N HCl. Each sample was then acetylated by the addition of trieethylamine and acetic anhydride and the amount of cAMP was determined by radioimmunoassay as previously detailed (Liggett et al., 1989).

Chemical Analysis

Chemical degradation and analysis of eumelanin and pheomelanin contents were performed and quantitated as previously reported (Ito and Fujita, 1985).

Molecular and biochemical mechanisms that switch melanocytes between the production of eumelanin or pheomelanin involve the opposing action of two intercelluar signalling molecules, α-melanocyte stimulating hormone and agouti signal protein. In this study, the physiological effects of agouti signal protein on melanosomal protein expression by eumelanogenic melanocytes in culture treated with purified recombinant agout signal protein were characterized. Following exposure of black melan-a murine melanocytes to agouti signal protein in vitro, pigmentation of the cells was markedly inhibited and the production of eumelanosomes was decreased significantly. In addition, the melanosomes that were produced became pheomelanosome-like in structure and chemical analysis showed that while eumelanin production was significantly decreased, the synthesis of pheomelanin was increased. Melanocytes treated with agout signal protein alone also exhibited time- and dose- dependent decreases in mRNA and protein content of several melanosomal proteins, including the melanogenic enzymes tyrosinase and tyrosinase-related proteins 1 and 2. Conversely, melanocytes exposed to α-melanocyte stimulating hormone alone exhibited an increase in tyrosinase mRNA and protein. Simultaneous addition of agouti signal protein and α-melanocyte stimulating

43

hormone at approximately equimolar concentrations produced responses similar to those elicited by α-melanocyte stimulating hormone alone. These data are consistent with the hypothesis that the effects of agouti signal protein on melanocytes are not mediated solely by inhibition of α-melanocyte stimulating hormone binding to its receptor, and provide a cell culture model to identify novel factors whose presence is required for pheomelanogenesis.

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SUBSTITUTE SHEET (RULE 26)

(SEQUENCE LISTING TO BE INSERTED)

48

What is claimed is:

1.   A purified biologically active peptide of the agouti signaling protein which has depigmenting activity.

2.   A purified biologically active peptide of the agouti signaling protein which has the following characteristics:

(a)   the peptide has depigmenting activity due to its ability to inhibit the production of at least one melanin;

(b)   the peptide has at least one region selected from the group consisting of:

(i)   at least one of a basic region derived from the full length agouti signaling protein or a portion thereof, and

(ii)   a cysteine rich region derived from the full length agouti signaling protein or a portion thereof,

(c)   the peptide has a minimum length of at least about 5 amino acid residues but has a maximum length selected from the group consisting of:

(i)   no more than about 131 amino acid residues;

(ii)   no more than about 109 amino acid residues;

(iii)   no more than about 50 amino acid residues;

(iv)   no more than about 20 amino acid residues; and

(v)   no more than about 10 amino acid residues; and

wherein said agouti signaling protein is optionally coupled to a molecule which would facilitate its transport into cells.

3.   The agouti signaling protein of claim 2 having a maximum length of no more than about 131 amino acid residues.

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4.   The agouti signaling protein of claim 3 having a maximum length of no more than about 109 amino acid residues.

5.   The peptide of claim 1, having the sequence of SEQ ID NO:1.

6.   The peptide of claim 1, having the sequence of SEQ ID NO:2.

7.   The peptide of claim 1, having the sequence of SEQ ID NO:3.

8.   The peptide of claim 1, having the sequence of SEQ ID NO:4.

9.   The peptide of claim 1, having the sequence of SEQ ID NO:5.

10.   The peptide of claim 1, having the sequence of SEQ ID NO:6

11.   The peptide of claim 1, having the sequence of SEQ ID NO:7.

12.   The peptide of claim 1, having the sequence of SEQ ID NO:8.

13.   The peptide of claim 1, having the sequence of SEQ ID NO:9.

14.   The peptide of claim 1, having the sequence of SEQ ID NO:10.

15.   The peptide of claim 1, having the sequence of SEQ ID NO:11.

16.   The peptide of claim 1, having the sequence of SEQ ID NO:12.

17.   The peptide of claim 1, having the sequence of SEQ ID NO:13.

18.   The peptide of claim 1, having the sequence of SEQ ID NO:14.

19.   The peptide of claim 1, having the sequence of SEQ ID NO:15.

20.   The peptide of claim 1, having the sequence of SEQ ID NO:16.

21.   The peptide of claim 1, having the sequence of SEQ ID NO:17.

22.   The peptide of claim 1, having the sequence of SEQ ID NO:18.

23.   The peptide of claim 1, having the sequence of SEQ ID NO:19.

24.   The peptide of claim 1, having the sequence of SEQ ID NO:20.

25.   The peptide of claim 1, having the sequence of SEQ ID NO:21.

26.   The peptide of claim 1, having the sequence of SEQ ID NO:22.

27.   The peptide of claim 1, having the sequence of SEQ ID NO:23.

28.   The peptide of claim 1, having the sequence of SEQ ID NO:24.

29.   The peptide of claim 1, having the sequence of SEQ ID NO:25.

30.   The peptide of claim 1, having the sequence of SEQ ID NO:26.

31.   The peptide of claim 1, having the sequence of SEQ ID NO:27.

32.   The peptide of claim 1, having the sequence of SEQ ID NO:28.

33.   The peptide of claim 1, having the sequence of SEQ ID NO:29.

51

34. A composition comprising the agouti signaling protein or biologically active peptide thereof and a pharmaceutically acceptable carrier.

35. A purified polyclonal antibody that specifically binds the peptide of claim 33.

36. A purified monoclonal antibody that specifically binds the peptide of claim 33.

37. A method of down-regulating in a subject a melanogenic enzyme involved in melanin synthesis, comprising administering to the subject an amount of the agouti signaling protein or a biologically active peptide thereof to down-regulate the melanogenic enzyme.

38. A method of reducing melanin synthesis in a subject, comprising administering to the subject an amount of the agouti signaling protein or a biologically active peptide thereof to reduce melanin synthesis.

39. A method of altering melanin synthesis in a subject, comprising administering to the subject an amount of a nucleic acid that hybridizes to a nucleic acid encoding an agouti signaling protein, whereby the agouti encoding nucleic acid is not transcribed and melanin synthesis is altered.

40. A method of treating a hyperpigmentary condition in a subject, comprising administering to the subject the agouti signaling protein or a biologically active peptide thereof.

41. A method of screening for inhibitors of melanin synthesis, comprising:

a) contacting a melanocyte culture with an amount of a putative inhibitor of melanin synthesis;

b) determining the amount of melanocyte enzyme present in the melanocyte culture from step a);

c) comparing the amount of melanocyte enzyme determined in step

d) to the amount of melanocyte enzyme in an uncontacted melanocyte culture; and

e) correlating a decrease in at least one melanocyte enzyme with an inhibitor of melanin synthesis.

Fig. 1
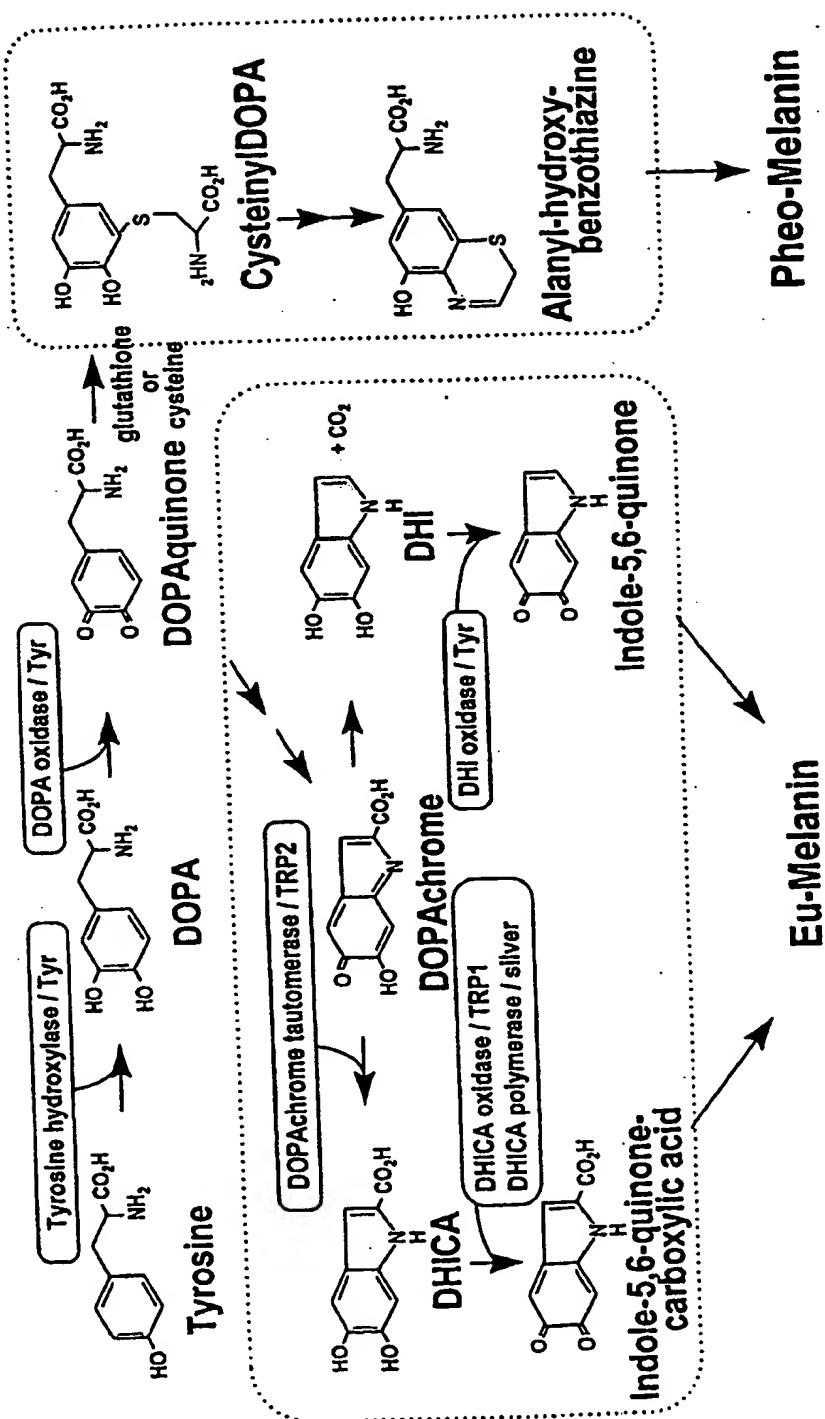
Figure 2
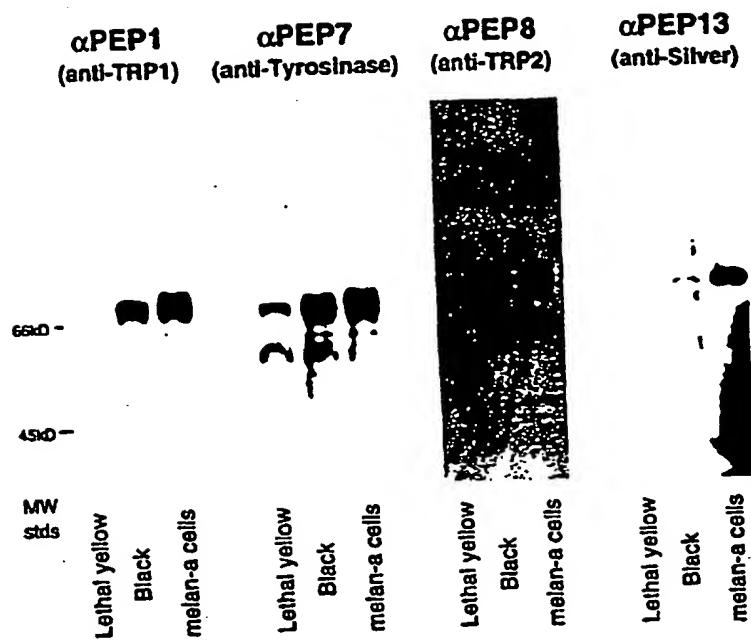
3/14

Fig. 3
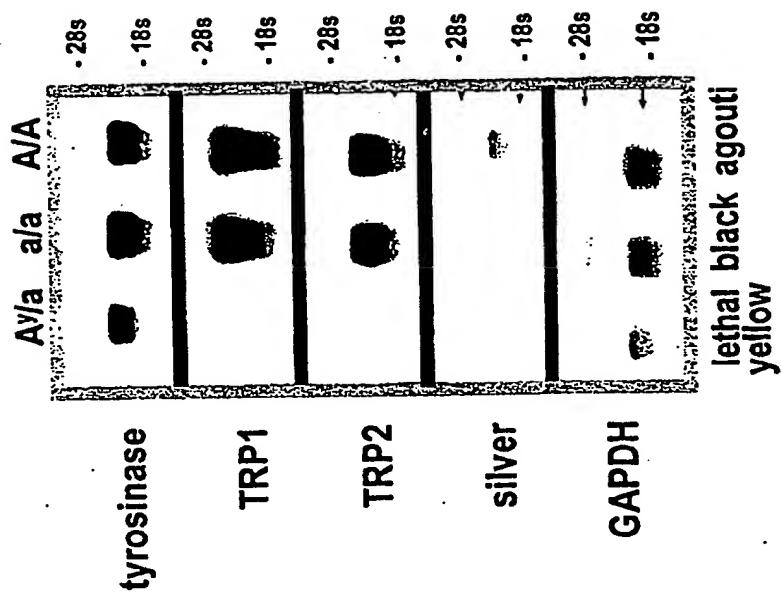
Figure 4
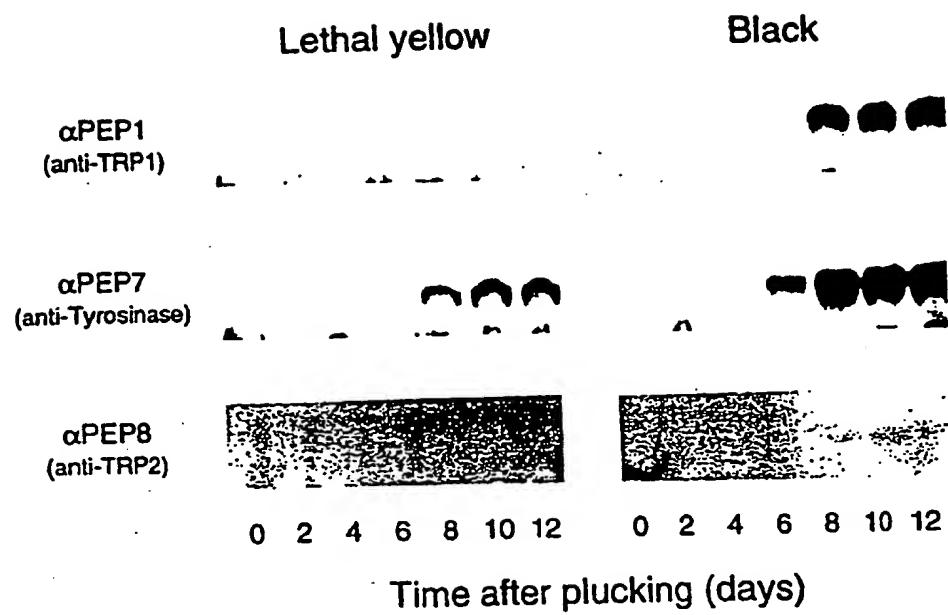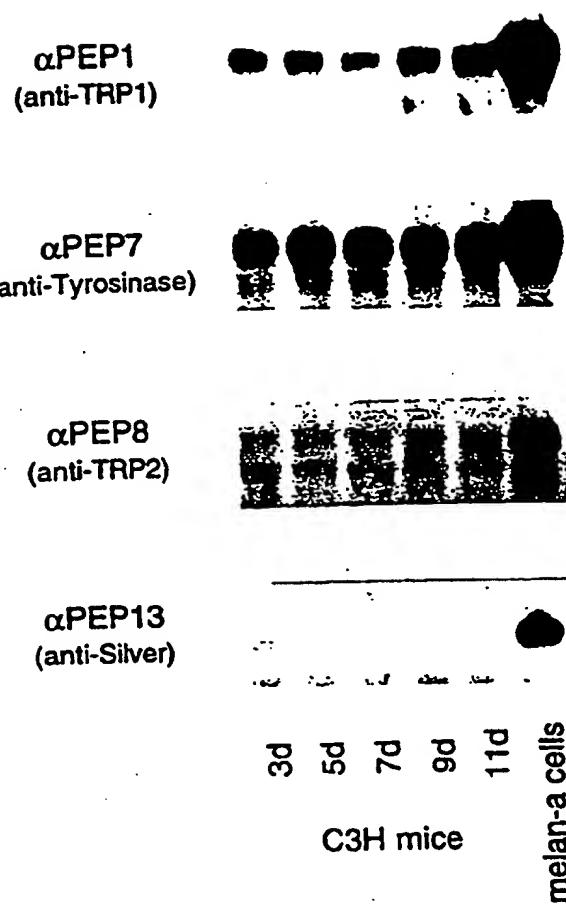
Lethal yellow   Black

αPEP1
(anti-TRP1)

αPEP7
(anti-Tyrosinase)

αPEP8
(anti-TRP2)

0 2 4 6 8 10 12   0 2 4 6 8 10 12

Time after plucking (days)

Figure 5

Fig. 6A

TRP1 (αPEP1)

tyrosinase (αPEP7)

TRP2 (αPEP8)

silver (αPEP13)

5 day   11 day
A/a a/a   A/a a/a

Figure 6B

Northern blotting

melan-a

1 day Rx

Figure 7A   −   +   agouti protein

10   1   0.1   0.01   nM

Tyrosinase

100   31   72   106   96   % control

GAPDH

10nM agouti protein

Figure 7B   −   +

1   2   4   days

Tyrosinase

100   46   3   -4   % control

GAPDH
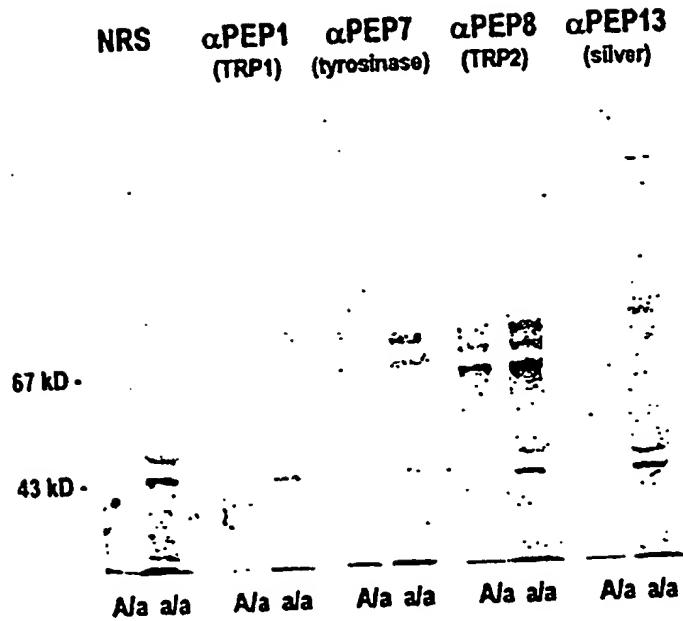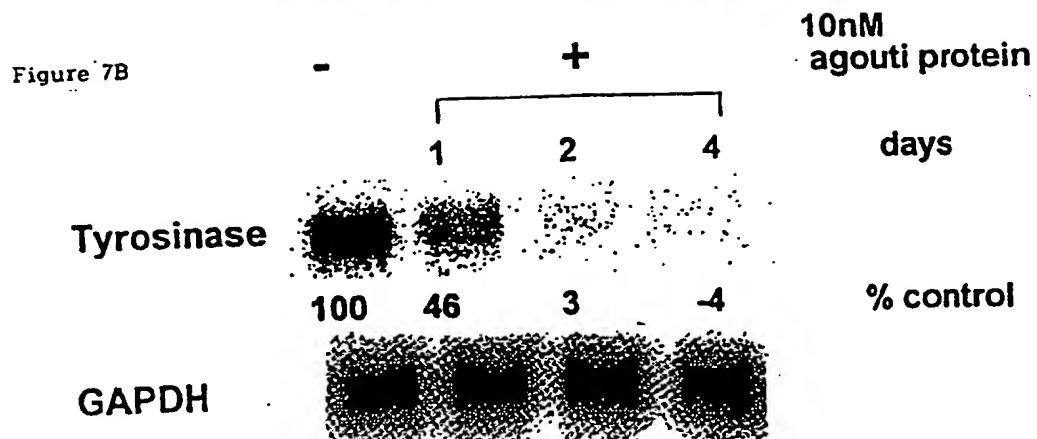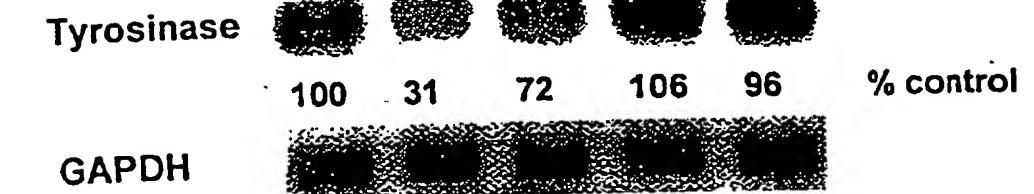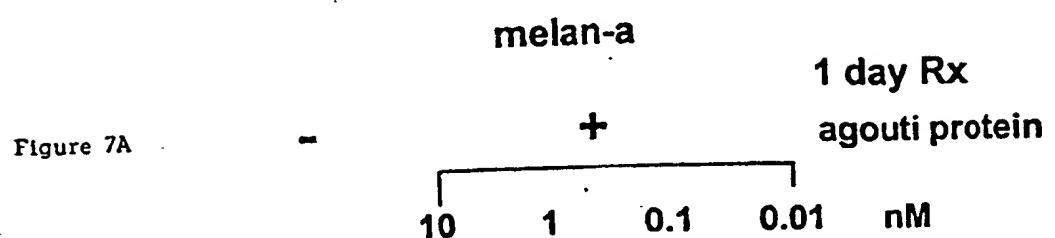
Ultrastructural study

melan-a

+ 5 days Rx agouti protein (10nM)
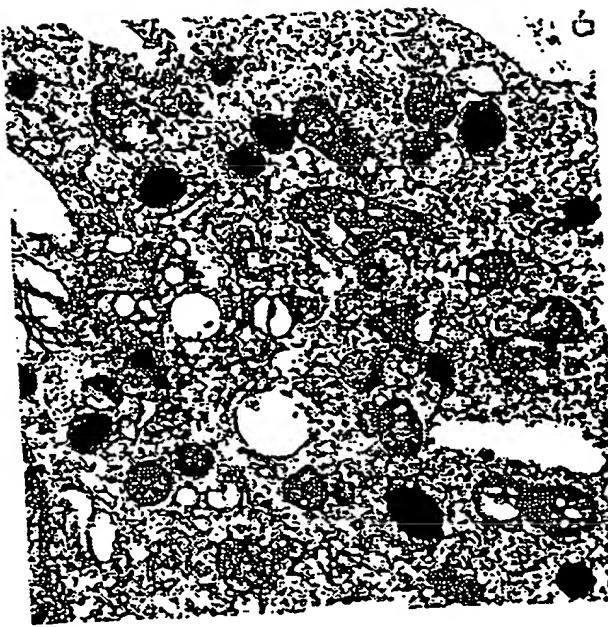
(x8000)

Figure 8A
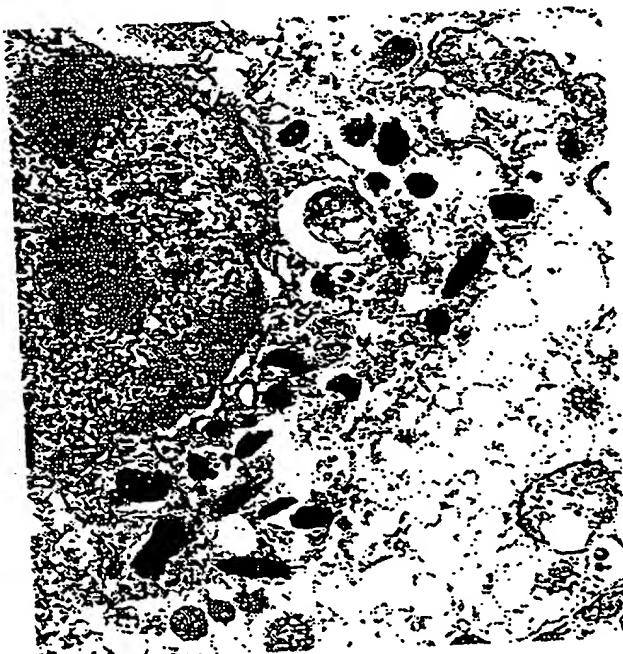
Figure 8B

Figure. 9

Northern blotting

melan-a   1 day Rx

-          +   agouti protein

10   1   0.1   0.01   nM

Tyrosinase

100   31   72   106   96   %control

TRP1

100   56   88   95   82   %control

TRP2

100   50   87   91   87   %control

MSH-R

100   86   97   100   79   %control

GAPDH
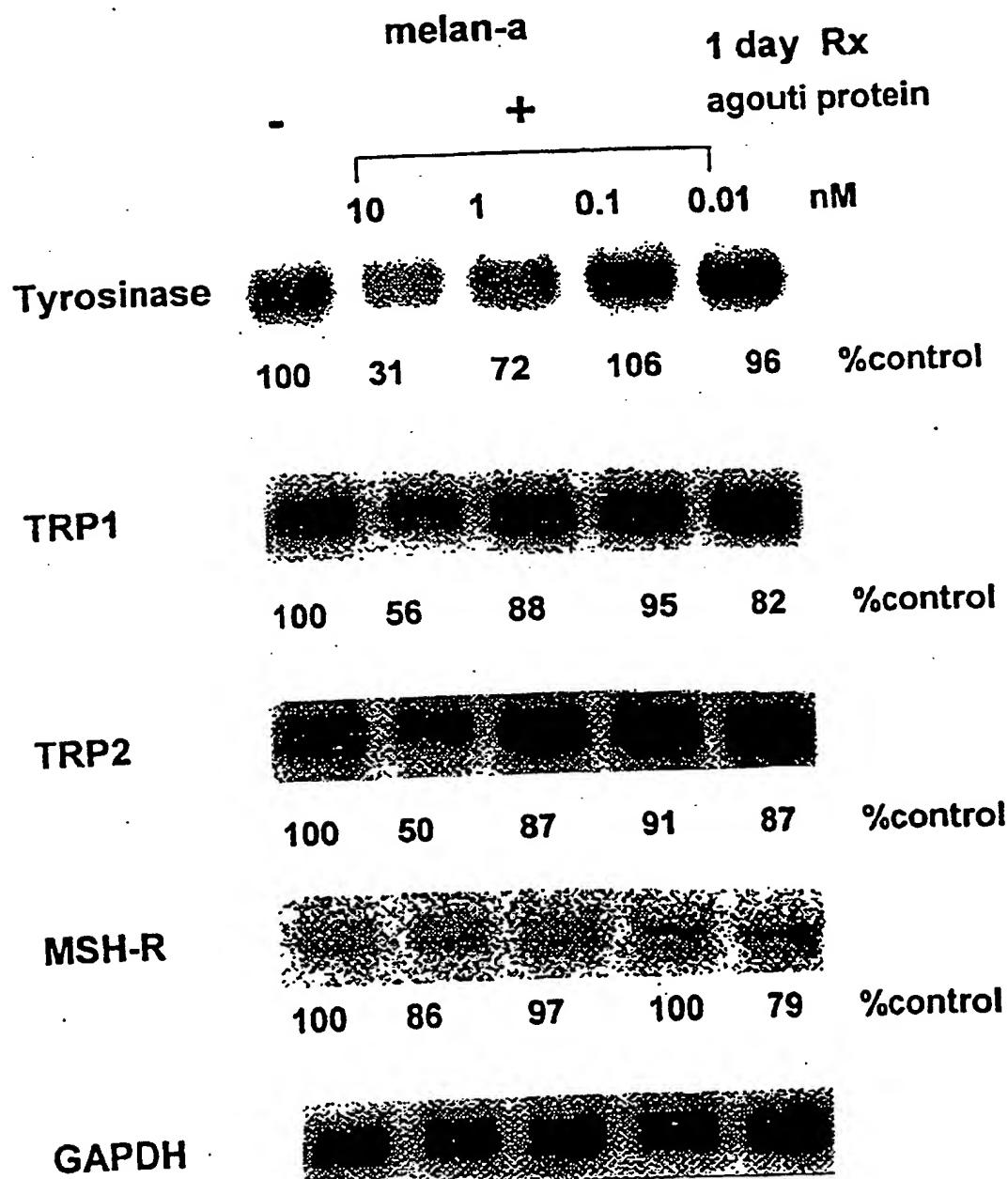
Figure 10

Northern blotting

	melan-a			5 days Rx

-	+	-	+	MSH (10nM)
-	-	+	+	agouti protein (10nM)

Tyrosinase

| 100 | 184 | 20 | 181 | %control |

TRP1

| 100 | 110 | 29 | 132 | %control |

TRP2

| 100 | 143 | 21 | 158 | %control |

MSH-R

| 100 | 101 | 98 | 116 | %control |

GAPDH
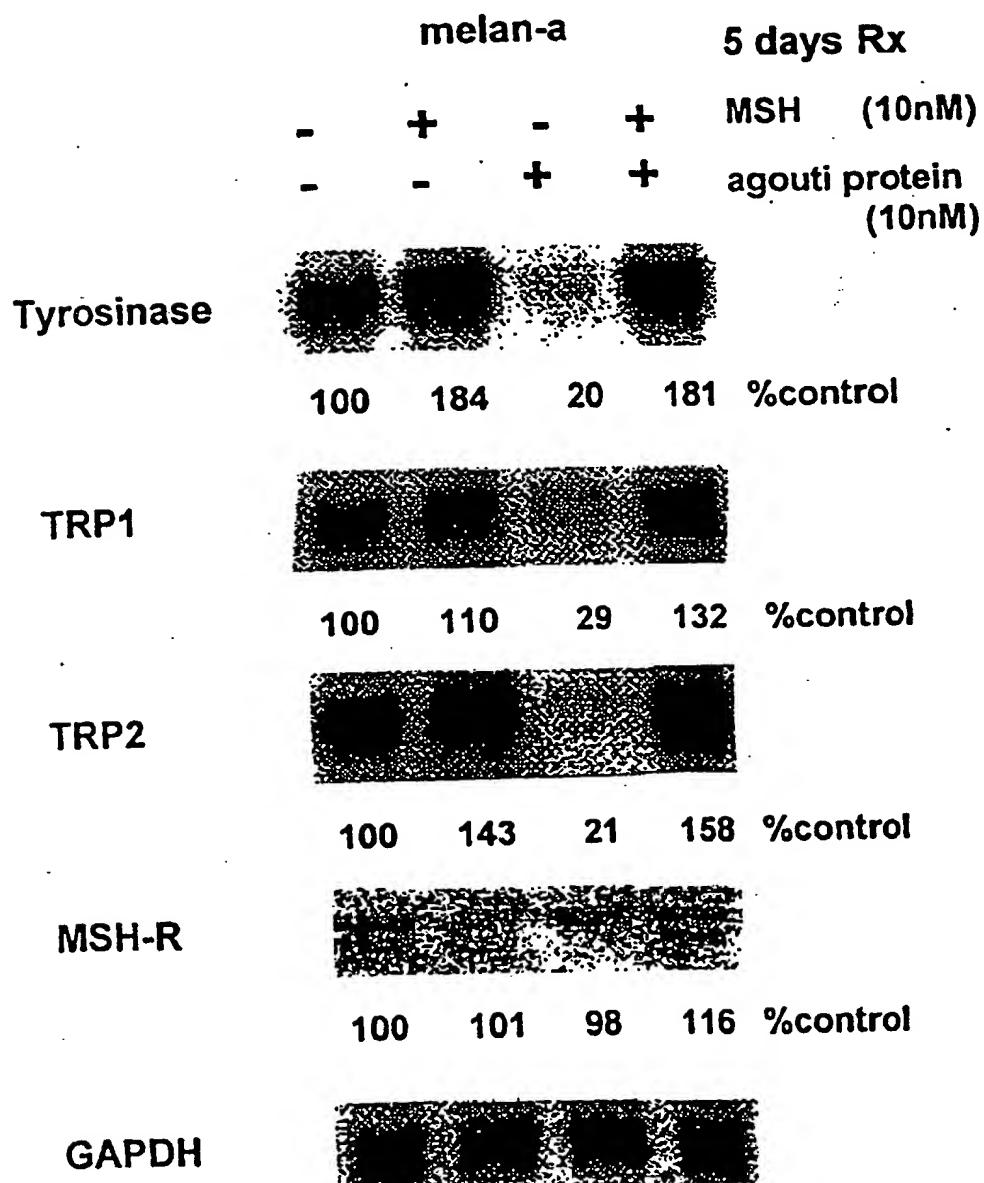
Figure 11

Metabolic labeling
and Immunoprecipitation

melan-a

5 days Rx

| − | + | − | + | MSH (10nM) |
| − | − | + | + | agouti protein (10nM) |

Tyrosinase

100   332   116   275   %control

TRP1

100   189   22   141   %control

TRP2

100   175   28   120   %control
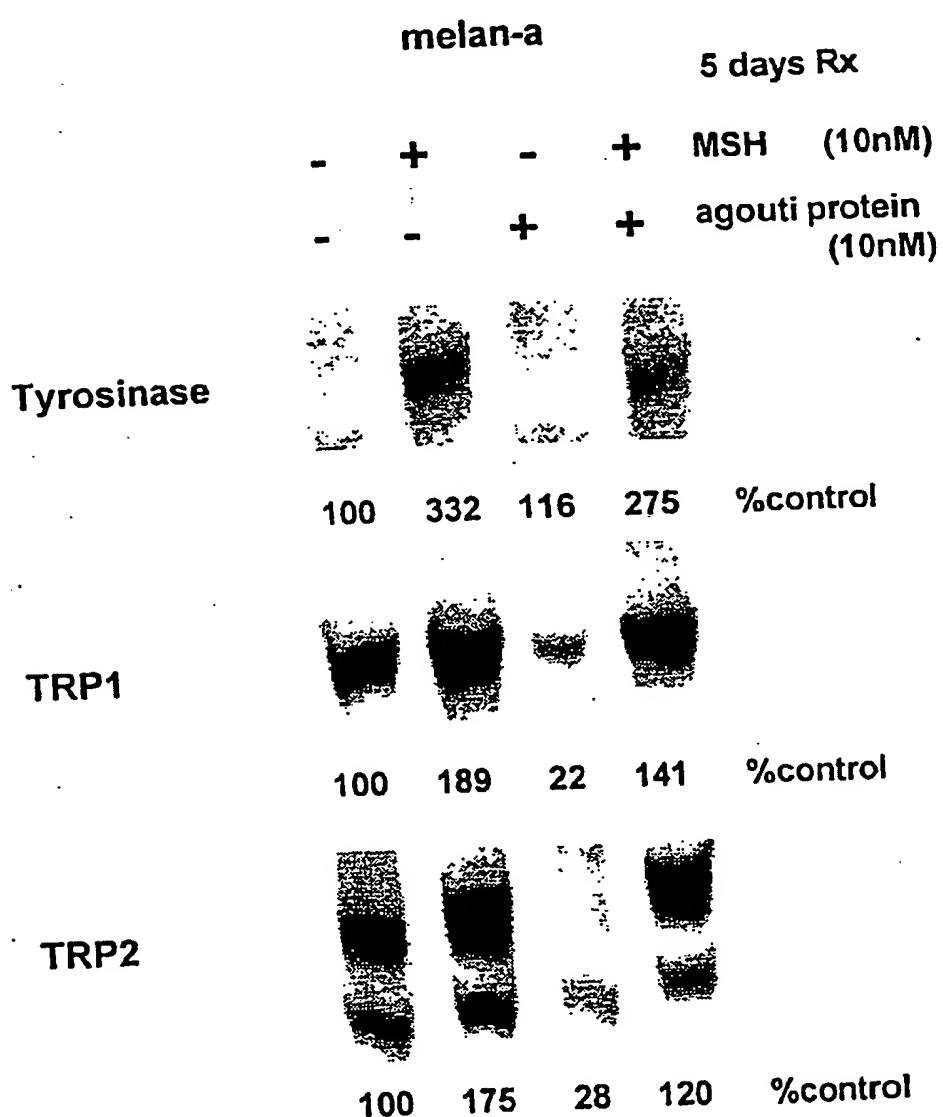
Figure 12

Western blotting

melan-a

5 days Rx

-	+	-	+	MSH (10nM)

-	-	+	+	agouti protein (10nM)

Tyrosinase

100   123   37   125   %control

TRP1

100   83   8   85   %control

TRP2

100   100   18   96   %control

Figure 13

Northern blotting

	B16F10			5 days Rx
-	+	-	+	MSH (10nM)
-	-	+	+	agouti protein (10nM)

Tyrosinase

| 100 | 217 | 134 | 210 | %control |

TRP1

| 100 | 157 | 62 | 182 | %control |

TRP2

| 100 | 163 | 77 | 173 | %control |

MSH-R

| 100 | 125 | 195 | 144 | %control |

GAPDH

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(54) Title: DEPIGMENTING ACTIVITY OF AGOUTI SIGNAL PROTEIN AND PEPTIDES THEREOF

(57) Abstract

The invention is an agouti signaling protein and peptides as well as pharmaceutical compositions thereof and their use in methods of inhibiting melanin production by melanocytes. The agouti signaling protein and peptides thereof are useful in cosmetics and in clinical prevention and treatment of hyperpigmentary conditions. Methods for screening peptides for melanogenesis inhibiting activity are also provided.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C07K14/47   A61K38/16   G01N33/68   C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C07K   A61K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J CELL SCI. , vol. 108, no. 6, June 1995, CAMBRIDGE, pages 2301-2309, XP000614781 T KOBAYASHI ET AL.: "Modulation of melanogenic protein expression during the switch from eu- to pheomelanoggenesis" see the whole document ---	1-41
A	CELL, vol. 71, 24 December 1992, NA US, pages 1195-1204, XP002024341 S J BULTMAN ET AL.: "Molecular characterization of the mouse agouti locus" see the whole document ---	1-41

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 1997	1 4. 02. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

Inte   nal Application No

PCT/US 96/10695

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 21, October 1994, WASHINGTON US, pages 9760-9764, XP002024342 H Y KWON ET AL.: "Molecular structure and chromosomal mapping of the human homolog of the agouti gene " see the whole document ---	1-41
A	File Medline, abstract no. 95276734 (1995) & B D Wilson et al., "Structure and functi on of ASP, the human homolog of the mouse agouti gene", Human Molecular Genetics, 4 (2), pages 223-230 (February 1995) XP002024343 cited in the application ---	1-41
A	File Medline, abstract no. 93194064 (1993) & M W Miller et al., "Cloning of the mouse agouti gene predicts a secreted protein ub iquitously expressed in mice carrying the lethal yellow mutation", Genes & Developme nt 7 (3), pages 454-467 (March 1993) XP002024344 cited in the application -----	1-41

1

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   **37-40**
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claims 37-40 refer to a method of treatment of the human body, the search was carried out and based on the alleged effects of the products.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

(72) Inventors: ORLOW, Seth, J.; 550 First Avenue, New York, NY 10016 (US). MANGA, Prashiela; 550 First Avenue, New York, NY 10016 (US).

(54) Title: SCREENING METHODS FOR COMPOUNDS THAT AFFECT MELANOGENESIS

(57) Abstract: Methods of screening for compounds that affect melanogenesis and the function of P protein in organisms, cells, or cell-free systems are provided. The invention further relates to the pharmacologic and cosmetic uses of such compounds to reduce or increase the synthesis of melanin in animal and human melanocytes and melanocyte-derived cells.

-1-

SCREENING METHODS FOR COMPOUNDS THAT AFFECT MELANOGENESIS

1.   Field of the Invention

The invention is in the fields of cell biology, drug discovery, and cosmetics. Methods of screening compounds that can affect P protein function are provided. The invention further relates to methods of using such compounds for the cosmetic and therapeutic reduction or increase of melanin content in human and animal cells.

2.   Background of the Invention

Melanin is a dark pigment found in plants and animals that protects against ultraviolet radiation and provides decoration in the skin, eyes, hair, and fur of animals (reviewed in Riley, P.A., 1997, Int. J. Biochem. Cell Biol. 11:1235-39). There are two different types of melanin: brown/black eumelanin and yellow/red pheomelanin. Melanocytes are cells of the epidermis specialized to produce melanin. A sophisticated intercellular signaling system determines whether an individual melanocyte will produce eumelanin or pheomelanin (reviewed in Brilliant, M.H. and Barsh, G.S., 1998, in *The Pigmentary System: Physiology and Pathophysiology*, 217-29, Oxford University, New York (Nordlund, J.J. et al., eds)).

Melanocytes synthesize melanin inside of specialized organelles called melanosomes (reviewed in Orlow, S.J., 1998, in *The Pigmentary System: Physiology and Pathophysiology*, 97-106, Oxford University, New York (Nordlund, J.J. et al., eds)). Melanosomes are formed by the fusion of two types of vesicles. One type of vesicle, called a premelanosome, apparently derives directly from either the smooth endoplasmic reticulum or the *trans*-Golgi network. The other type of vesicle derives from the *trans*-Golgi network. Each of these types of vesicles contributes proteins to the melanosome necessary for its function.

Defects in the production of melanin result in pigmentation deficiencies such as albinism. Genetic analysis of abnormally pigmented strains of mice has identified more than 60 genes necessary for the normal production of melanin (reviewed in Silvers, W.K., 1979, *The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction*, Springer-Verlag, Basel). One of these genes encodes the enzyme tyrosinase. Tyrosinase protein is a multi-functional enzyme that catalyzes several steps in the production of melanin; tyrosinase activities include the rate-limiting steps of converting tyrosine to dihydroxyphenylalanine (DOPA), and DOPA to dopaquinone (reviewed in Lerner, A.B., and Fitzpatrick, T.B., 1950, Physiol. Rev. 30:91-126), as well as the oxidation of 5,6-dihydroxyindole to 5,6-indolequinone (Korner and Pawelek, 1982, Science 217:1163-1165). Both humans and mice lacking tyrosinase activity suffer a severe form of albinism.

Two tyrosinase-related proteins (TRP-1, encoded by the mouse *brown* gene, and TRP-2, encoded by the mouse *slaty* gene) also are important for melanogenesis (reviewed in Hearing, V.J., 1993, Am. J. Hum. Genet. 52:1-7). Each of the TRP proteins shares about 40% sequence identity with tyrosinase and with each other. Each of these three enzymes

5   (tyrosinase, TRP-1 and TRP-2) is predicted to contain one transmembrane domain. Together, they form a high molecular weight complex associated with the melanosomal membrane (Orlow, S.J., *et al.*, 1994, J. Invest. Dermatol. 103:196-201).

Another protein that is important for the production of melanin is the P protein. In mice, it is the product of the pink-eye dilution (p) gene. In humans, it is the product of the P

10 · gene. Humans lacking P protein function suffer from type II oculocutaneous albinism (Durham-Pierre, D., *et al.*, 1994, Nature Genet. 7:176-79). *p*-null mice produce significantly less melanin than wild-type mice (Silvers, above). A wild-type human P gene, but not a mutant human P gene, can complement the hypopigmented phenotype of *p*-null mouse melanocytes (Sviderskaya, E.V., *et al.*, 1997, J. Invest. Dermatol. 108:30-34). P protein is

15 apparently needed for the production of eumelanin, but not of pheomelanin (Lamoreux, M.L., *et al.*, 1995, Pigment Cell Res. 8:263-70).

The P protein is predicted to contain 12 membrane spanning domains (Gardner, J.M., *et al.*, 1992, Science 257:1121-24). Consistent with this prediction, the P protein is found associated with the surface of the melanosome (Rosemblat, S., *et al.*, 1994, Proc. Natl. Acad.

20 Sci. USA 91:12071-75), which is the same membrane surface thought to be associated with the high molecular weight tyrosinase-containing complex described above.

Several authors have suggested that P protein acts as a tyrosine transporter by pumping tyrosine into the melanosome where it is converted into melanin by tyrosinase activity (*see, e.g.*, Rinchik, E.M., *et al.*, 1993, Nature 361:72-76). First, the P protein bears

25 some resemblance to transport proteins found in prokaryotes. Second, cultured *p*-null mutant mouse melanocytes, which produce much less melanin than cultured wild-type mouse melanocytes, make increased levels of melanin when high concentrations of tyrosine are added to the cells' growth medium (Sviderskaya, E.V., *et al.*, above; Rosemblat, S. *et al.*, 1998, Exp. Cell Res. 239:344-52). However, contradicting this suggestion, it has been found

30 that tyrosine uptake by melanosomes is virtually the same in *p*-null and wild-type melanocytes (Gahl, W.A. *et al.*, 1995, Pigment Cell Res. 8:229-233). This observation has led other authors to hypothesize that P protein is necessary for the transport into melanosomes of some other small molecule necessary for melanogenesis (summarized in Brilliant, M.H. and Barsh, G.S., 1998, above).

35 Other authors have speculated that P protein plays a structural role in melanosomes (Lamoreux, M.L., *et al.*, above). The integrity of melanosomes is compromised in cells lacking P protein. Tyrosinase activity, and therefore melanin production, is greatly decreased

-3-

in these defective melanosomes. Specifically, tyrosinase activity levels in melanocyte extracts of skin and eyes from p-null mice are lower than such extracts from wild-type mice (Lamoreux, M.L., et al., above; Chiu, E., et al, 1993, Exp. Eye Res. 57:301-05). Moreover, levels of tyrosinase, TRP-1 and TRP-2 proteins are lower in p-null tissue extracts than in

5   wild-type extracts (Rosemblat, S., et al., 1998, above). Additionally, a much greater percentage of tyrosinase, TRP-1, and TRP-2 proteins are found in their monomeric forms, rather than as part of a high molecular weight complex, in p-null tissue extracts than in wild-type extracts (Lamoreux, M.L., et al., above; Chiu, E., et al., above), and tyrosinase, TRP-1, and TRP-2 are all rapidly degraded in the ocular tissue of p-null mice (Chiu, E., et al.,

10   above). Finally, several authors have observed that melanosomes in p-null tissues and cultured melanocytes are abnormal (Russell, E.S., 1949, Genetics 34:146-66; Rosemblat, S. et al., 1998, above). In p-mutant melanocytes from mouse eye, very few melanosomes are observed (Orlow, S.J. and Brilliant, M.H., 1999, Exp. Eye Res. 68:147-54). In cultured mutant melanocytes, a greater than normal number of melanosomes is present, but they are smaller

15   than those seen in wild-type melanocytes (Rosemblat, S. et al., 1998, above).

Thus, although P protein is known to be critical for the production of normal amounts of melanin in the skin, hair and eyes, the function of the P protein in this process has remained elusive. Instead, researchers have looked to other molecular targets for inhibition studies. For example, tyrosinase's well-characterized enzymatic activity, amenability to

20   biochemical analysis, and pivotal role in melanogenesis have made it an inviting target for inhibition studies (see, e.g., Tasaka, K., et al., 1998, Meth. Find. Exp. Clin. Pharmacol. 20:99-109; Iida, K., et al., 1995, Planta Med. 61:425-28; Reish, O., et al., 1995, Am. J. Hum. Genet. 57:127-32; Shirota, S., et al., 1994, Biol. Pharm. Bull. 17:266-69; Kameyama, K., et al., 1989, Differentiation 42:28-36). Researchers have also focused on the effects of

25   intercellular signaling molecules on melanogenesis (see, e.g., Furumura, M. et al., 1998, Proc. Natl. Acad. Sci. USA 95:7374-78; Sakai, C., et al., 1997, EMBO J. 16:3544-52; McLeod, S.D. et al., 1995, J. Endocrinol. 146:439-47).

3..   Summary of the Invention

The present invention provides novel screens for the identification of compounds that

30   inhibit or increase melanogenesis in melanogenic cells. The development of these assays is based, in part, on the discovery that some compounds that inhibit melanogenesis do so by causing a mislocalization of tyrosinase, the key enzyme in melanin synthesis.

The P protein is a pivotal target for compounds and drugs to decrease or increase pigmentation of the skin, hair and/or eyes. Accordingly, in one aspect, the present invention

35   provides, for the first time, screens for compounds that inhibit or enhance P protein function based, in part, on the discovery that P protein function is required for proper cellular localization of tyrosinase and other melanosomal proteins and is required for both full

-4-

tyrosinase activity and melanogenesis in melanogenic cell types such as, for example, melanocytes and melanoma cells.

Wild-type melanogenic cells target tyrosinase primarily to melanosomes. Some tyrosinase is also secreted by these cells. P protein-compromised melanogenic cells

5   mislocalize tyrosinase. They secrete significantly more tyrosinase than wild-type melanogenic cells, and also contain tyrosinase in non-melanosomal vesicles. Tyrosinase that is secreted from melanogenic cells, regardless of whether the cells have normal or inhibited P protein function, is enzymatically active in the growth or incubation medium, where it can convert tyrosine into melanin.

10   In one aspect, the present invention provides a method of screening for compounds that inhibit melanogenesis in melanogenic cells, comprising incubating these cells in medium containing a compound to be tested, and identifying compounds that cause a change in the cellular localization of tyrosinase in these cells. Mislocalization of tyrosinase can indicate inhibition of melanogenesis.

15   In a still further aspect, the present invention provides methods of screening for compounds that increase melanogenesis in melanogenic cells, comprising incubating melanogenic cells in medium containing a compound to be tested, and identifying compounds that cause a decrease in the amount of tyrosinase secreted by the cells relative to the amount of tyrosinase retained by the cell, wherein such relative decrease in the amount of tyrosinase

20   secreted indicates that the compound is a candidate for a compound that increases melanogenesis.

Another aspect of the invention is based, in part, on the discovery that non-melanogenic cells can be made to produce active tyrosinase by transfecting them with a heterologous tyrosinase-encoding gene. The tyrosinase activity of these cells is dramatically

25   increased by cotransfection with a heterologous P protein-encoding gene. Maximal tyrosinase activity in these cells is therefore dependent upon P protein function. When cells expressing both heterologous tyrosinase and heterologous P protein are treated with drugs that inhibit P protein function such as, for example, imipramine, the tyrosinase activity of these cells is reduced to that of cells expressing heterologous tyrosinase alone. Imipramine and

30   other drugs that inhibit P protein function do not otherwise affect tyrosinase activity in cells that express heterologous tyrosinase but that do not express heterologous P protein.

Accordingly, in a further aspect, the present invention provides methods of screening for compounds that affect (e.g., either inhibit or increase) P protein function in cells that do not ordinarily express tyrosinase and/or P protein, comprising manipulating these cells so that

35   they express both tyrosinase and P protein, and treating the cells with a compound to be tested. The tyrosinase activity of these cells is measured. Compounds that affect (e.g., inhibit or increase) the tyrosinase activity of these cells, but that do not affect the tyrosinase

activity of cells expressing tyrosinase alone, are identified as compounds that affect tyrosinase in a P protein dependent manner.

In a further aspect, the present invention provides methods for modeling chemical compounds known to affect or mimic the function of P protein. Analogs of the modeled compound are selected or designed, and screened for the ability to affect P protein function. By using analogs of a compound known to affect or mimic P protein function, new and better compounds that affect or mimic P protein function can be discovered using the methods of the present invention.

In a still further aspect, the present invention provides methods for using, in medicinal and cosmetic compositions, compounds that affect or mimic the function of P protein, thereby treating a disease, condition, or disorder involving the production (underproduction or overproduction) of melanin.

4.   Brief Description of the Figures

FIG. 1. Tyrosinase activity in media from cultured melanocytes.   Melan-a melanocytes cultured from black mice, and melan-p melanocytes cultured from mice lacking a P gene transcript, were separately cultured in DMEM containing low (0.03 mM) or high (0.3 mM) tyrosine for the indicated time period. The activity of tyrosinase was determined at specific time intervals in media from melanocytes. The medium was dialyzed prior to determining the enzyme activity, which is expressed as cpm of tritiated water generated per hour. ▲ Melan-a, high tyrosine; • melan-a, low tyrosine; Δ melan-p1, high tyrosine; ° melan-p1, low tyrosine. Increasing tyrosinase activity in the media removed from melan-p cells, which have no P protein transcripts, grown in the presence of 0.03 mM tyrosine, reflects an increased secretion of tyrosinase by these cells. In contrast, melan-a cells, which represent wild-type melanocytes, secrete significantly less tyrosinase into the media. Growing melan-p cells in the presence of high tyrosine partially alleviated the P-deficient phenotype.

FIG. 2. Tyrosinase activity in cell extracts and media from melan-A cells. Cultured melan-a melanocytes were incubated for 48 hours in the presence of benztropine, imipramine, nitroquipazine, or left untreated. Incubation media or cell extracts were assayed for tyrosine hydroxylase activity, as in FIG. 1. Column 1, untreated melanocytes; Column 2, melanocytes treated with benztropine; Column 3, melanocytes treated with 10,11-Dihydro-n,n-dimethyl-5H-dibenz[b,f]azepine-5-propanamine (imipramine); Column 4, melanocytes treated with 6-Nitro-2-(1-piperazinyl)-quinoline maleate (nitroquipazine). In FIG. 2a (left), tyrosine hydroxylase activity of melan-a cell extracts is measured in cpm [^{3}H]H$_2$O/60 micrograms protein/hr. In FIG. 2b (right), tyrosine hydroxylase activity in media from melan-a cells is measured in cpm [^{3}H]H$_2$O/hr normalized to the amount of cell extract protein. The tyrosine hydroxylase activity of extracts from melan-a cells incubated with benztropine (column 2 in FIG. 2a.) and nitroquipazine (column 4 in FIG. 2a) is higher than that seen in

untreated cells. The extracts from cells treated with imipramine (column 3 in FIG. 2a) show a
reduced activity. The effects of the drugs on the enzyme activity of the cell extracts is not
reflected in the activity of the enzyme secreted into the media. While benztropine has little
effect on activity (column 2 in FIG. 2b), imipramine (column 3 in FIG. 2b) and nitroquipazine
5   (column 4 in FIG. 2b) cause a significant increase in activity in the media.

FIG. 3.   Relative tyrosinase activity in transfected COS cells.   COS cells were
transfected with two doses of the vector alone (V+V), one dose of the vector alone and one
dose of the vector carrying a tyrosinase-encoding gene (V+T), one dose of the vector alone
and one dose of the vector carrying a P protein-encoding gene (V+P), or one dose each of the
10   vectors carrying a tyrosinase-encoding gene and a P protein-encoding gene (T+P). Equal
quantities of cell extract protein were assayed for tyrosine hydroxylase activity. Relative
activities shown are calculated as the activity of the test sample divided by the activity of the
V+T sample. The introduction of an expression plasmid carrying the tyrosinase gene (V + T)
results in tyrosine hydroxylase activity in COS cells. This activity is the direct result of the
15   tyrosinase-encoding plasmid, since transfection with the expression vector (V + V) alone does
not generate any tyrosine hydroxylase activity. The tyrosine hydroxylase activity in cells
carrying the tyrosinase-encoding plasmid can be increased almost 4-fold by co-transfection
with the P gene expression plasmid (T + P). This increase is the result of an interaction
between tyrosinase and P protein, since the introduction of P (V + P) without tyrosinase
20   generates no tyrosine hydroxylase activity.

FIG. 4. Tyrosinase activity in transfected COS cells. COS cells transfected with a
vector carrying a tyrosinase-encoding gene, or with a first vector carrying a tyrosinase-
encoding gene and with a second vector carrying a P protein-encoding gene as in FIG. 3,
were treated with benztropine, imipramine, nitroquipazine, or left untreated, as in FIG. 2. Cell
25   extracts were prepared as in FIG. 3 The tyrosine hydroxylase activity of cell extracts was
determined as in FIG. 1 as a measure of tyrosinase activity. Column 1, untreated
transfectants; Column 2, transfectants treated with benztropine; Column 3, transfectants
treated with imipramine; Column 4, transfectants treated with nitroquipazine.   Tyrosine
hydroxylase activity is measured in cpm [^{3}H]H$_2$O/60 micrograms protein/hr.   Cells co-
30   transfected with a tyrosinase-encoding gene and a P protein-encoding gene (T + P) show a
higher tyrosine hydroxylase activity than cells transfected with a tyrosinase-encoding gene
alone (V + T) (column 1). This effect is not altered by incubation of cells in the presence of
benztropine (column 2) or nitroquipazine (column 4). The presence of imipramine, however,
abolishes the effect of P protein while appearing to have little effect on the activity in the cells
35   with tyrosinase alone (column 3).

FIG. 5. Levels of secreted tyrosinase are elevated in melan-p1 and are reduced by
inhibition of cysteinyl proteases. (a) Melan-p1 cells incubated in low (0.03 mM) tyrosine and

-7-

high (0.3 mM) tyrosine (TYR) were treated for 48 hours with increasing concentrations of the protease inhibitor E64 (μM). The tyrosinase activity in the media is expressed as a percentage of total activity in the extract and medium. (b) The concentration of melanin was determined by solubilizing the cell pellet and measuring the absorbance at 470 nm.

5   FIG. 6. Ultrastructure of cultured melanocytes. The peri-nuclear area of melan-a (A) and melan-p1 (B) melanocytes demonstrating the Golgi apparatus (G). Melanosomes in the melan-a cell are of stage I, II, III, and predominantly stage IV (arrows). Melanosomes in melan-p1 cells are predominantly stage I and II with an occasional early stage III (arrowheads); no stage IV melanosomes were observed. BAR = 1.0 micron.

10   FIG. 7. Ultrastructure of cultured melanocytes processed for DOPA histochemistry. The perinuclear area of melan-a (A) and melan-p1 (B) melanocytes demonstrating the Golgi apparatus with DOPA reaction product in the cisternae and 50 nm vesicles of the TGN (G). The 50 nm vesicles are confined to the TGN in the melan-a cells and radiate away from the TGN in melan-p1 cells (arrowheads), and can be observed in close proximity to the plasma
15   membrane (inset). Occasional stage III melanosomes are noted (arrows). BAR = 1.0 micron.

FIG. 8. Acid phosphatase targeting in melan-A and melan-P cells. Acid phosphatase activity was measured in fractionated membranes from melan-a (squares) and melan-p cells (circles) as described below in the Examples (Section 10). FIG. 8A = small granule fractions. FIG. 8B = large granule fractions.

20   FIG. 9. β-galactosidase targeting in melan-A and melan-P cells. β-galactosidase activity was measured in fractionated membranes from melan-a (squares) and melan-p cells (circles) as described below in the Examples (Section 10). FIG. 9A = small granule fractions. FIG. 9B = large granule fractions.

FIG. 10. β-hexosaminidase targeting in melan-A and melan-P cells. β-
25   hexosaminidase activity was measured in fractionated membranes from melan-a (squares) and melan-p cells (circles) as described below in the Examples (Section 10). FIG. 10A = small granule fractions. FIG. 10B = large granule fractions.

FIG. 11. β-glucosidase targeting in melan-A and melan-P cells. β-glucosidase activity was measured in fractionated membranes from melan-a (squares) and melan-p cells
30   (circles) as described below in the Examples (Section 10). FIG. 11A = small granule fractions. FIG. 11B = large granule fractions.

FIG. 12. β-glucuronidase targeting in melan-A and melan-P cells. β-glucuronidase activity was measured in fractionated membranes from melan-a (squares) and melan-p cells (circles) as described below in the Examples (Section 10). FIG. 12A = small granule
35   fractions. FIG. 12B = large granule fractions.

-8-

5.   Detailed Description of the Invention

The invention is based, in part, on the discovery that compounds that cause melanogenic cells to mislocalize tyrosinase (e.g., to increase the amount of tyrosinase secreted or the amount of tyrosinase found in non-melanosomal vesicles) also inhibit

5   melanogenesis. For purposes of the present invention, the term "melanogenic cells" is defined as cells that contain pigmented melanosomes (e.g., melanocyte cells and melanoma cells). Melanogenic cells can include, for example, melanogenic cells that express heterologous melanosomal proteins. For example, in preferred embodiments, the coding sequence or sequences of the P protein gene, tyrosinase gene, TRP-1 gene, and/or TRP-2

10   gene in a mouse melanogenic cell can be mutated or deleted, and the cell engineered to express instead the corresponding coding sequence of the human P protein gene, tyrosinase gene, TRP-1 gene, and/or TRP-2 gene.

Another aspect of the present invention is based, in part, on the discovery that the P protein is necessary to correctly localize tyrosinase predominantly to the membrane of

15   melanosomes.

Yet another aspect of the present invention is based on the finding that melanocytes treated with compounds that inhibit P protein function accumulate reduced amounts of intracellular melanin, and secrete increased amounts of tyrosinase into the growth medium.

Still another aspect of the present invention relates to the discovery that, in the

20   presence of the P protein, the enzymatic activity of tyrosinase protein in cultured cells is augmented.

Accordingly, the present invention provides novel methods of screening for compounds that inhibit melanogenesis. Compounds identified using the methods of the present invention are useful for treating diseases and cosmetic defects associated with the

25   underproduction or overproduction of melanin.

In another aspect, the present invention relates to the discoveries that wild-type melanogenic cells with normal P protein function secrete some tyrosinase, and that compounds that increase secretion of tyrosinase in a P protein dependent manner also inhibit melanogenesis. Accordingly, the present invention provides novel methods of screening for

30   compounds that increase melanogenesis by increasing the function of P protein. For purposes of this application, compounds that increase the function of P protein and compounds that decrease the function of P protein are collectively referred to herein as "compounds that affect the function of P protein." Still another aspect of the invention is a method of screening for compounds that increase melanogenesis by mimicking the function of

35   P protein. For purposes of the invention, "compounds that mimic the function of P protein" are compounds that are not P proteins, yet when they are administered to, or incubated with, melanogenic cells that do not contain P protein, they serve to restore at least in part the

correct targeting of tyrosinase to the melanogenic membrane. Melanogenic cells that do not contain P protein may be cells that do not express P protein transcripts (such as melan-p cells, described herein) or melanogenic cells that do not express a functional P protein gene product.

5   5.1   Methods of Screening for Inhibitors or Inducers of Melanogenesis
                    5.1.1   Methods of Screening for Inhibitors of Melanogenesis Using
                            Melanogenic Cells

In order for melanogenic cells to engage in robust melanogenesis, they must target their tyrosinase predominantly to the melanosomal membrane. Consequently, in one aspect,

10   the methods of the present invention entail screening for compounds that cause melanogenic cells to mislocalize tyrosinase. P protein function is necessary for the correct cellular localization of tyrosinase. Therefore, in another aspect, the methods of the present invention entail screening for compounds that inhibit P protein function, thereby causing melanogenic cells to mislocalize tyrosinase. Such methods are based, in part, on the discovery that

15   cultured melanocytes that have been genetically altered to eliminate P protein function secrete significantly more tyrosinase into the growth medium than wild-type melanocytes. Compounds, such as, e.g., imipramine, that reduce or eliminate P protein function will have the same effect. Thus, the cellular mislocalization of tyrosinase by cells treated with a test compound indicates that the test compound inhibits melanogenesis. Mislocalization of

20   tyrosinase resulting in secretion can be detected initially by assaying either the level of tyrosinase activity in the medium or cells, or the level of tyrosinase protein in the medium or cells. Test compounds that cause an increase in secretion of tyrosinase, or a decrease in intracellular tyrosinase, are candidates for compounds that inhibit melanogenesis by inhibiting P protein function. Such candidate compounds can be further investigated for their effect on

25   melanogenesis, and/or for their effects in both the presence and absence of P protein, as described more fully below. If the effect of the candidate compound depends upon the presence of P protein, then the compound inhibits the function of P protein.

Because growing P-protein-deficient melanocytes in the presence of high levels of tyrosinase in the medium can partially rescue the P-protein-deficiency, it is preferable, but not

30   necessary, that a screen for inhibitors of melanogenesis is carried out in the presence of low amounts of tyrosine in the media, e.g., 0.01-0.05 µm tyrosine, more preferably 0.014-0.03 µm tyrosine.

5.1.1.1 Methods of Screening for Inhibitors of Melanogenesis Using Assays for Tyrosinase activity

35   Wild-type melanogenic cells grown in in vitro culture will synthesize melanin inside of melanosomes as they do in vivo. In these cultured cells, tyrosinase is found predominantly in the melanosomal membrane, although some tyrosinase is also secreted. The tyrosinase that

-10-

is found in the melanosomal membrane is held in place by a C-terminal transmembrane domain and has its active site disposed toward the melanosomal lumen. By contrast, in melanogenic cells inhibited for melanogenesis through either a mutation in P protein or a compound that inhibits P protein function, tyrosinase will be mislocalized. A significantly

5   greater fraction of the cells' tyrosinase is secreted from the cells into the growth or incubation medium. Additionally, the secreted tyrosinase polypeptide will be shorter than that found in wild-type cells because it lacks its C-terminal membrane anchor. The secreted tyrosinase, however, is enzymatically active in the growth or incubation medium where it can synthesize melanin from extracellular tyrosine. Consequently, tyrosine-containing growth or incubation

10   media from melanogenic cells that have been inhibited for melanogenesis will turn dark. The higher the concentration of tyrosine in the medium, the darker the medium becomes, and the higher the concentration of tyrosinase in the medium, the faster the medium darkens. Because melanogenic cells that are not inhibited for melanogenesis secrete significantly less tyrosinase, the tyrosine-containing growth or incubation media in which they are cultured will

15   not become as dark.

       This discovery can be used in a novel method of screening to identify compounds that inhibit or modulate melanogenesis. Melanogenic cells are grown in culture or incubated in medium containing tyrosine. The cells are treated with a test compound. If the test compound causes tyrosinase to be mislocalized and secreted from the treated cells, then

20   tyrosine in the medium will be converted into melanin, darkening the medium. An assay is used wherein the color of the medium is compared to the color of the medium of the melanogenic cells grown or incubated under similar conditions but without the test compound (a control medium). If the medium of the cells treated with the test compound turns darker than the control medium, then the test compound is identified as candidate for a compound

25   that inhibits melanogenesis.

       More typically, in order to obtain at least semi-quantitative data, the media from the cells is first filtered, centrifuged and/or dialyzed prior to assay for tyrosinase activity. These types of treatments remove potentially confounding factors such as cells or particulate matter (e.g., melanosome or shed membranes) containing tyrosinase that could compete for

30   substrate, and/or remove excess free tyrosine that might compete with labeled substrate. Any of a number of alternative ways of measuring tyrosinase can be carried out, such as by using any of the enzymatic tyrosinase activities including but not limited to converting tyrosine to dihydroxyphenylalanine (DOPA), DOPA to dopaquinone, and oxidation of 5,6-dihydroxyindole to 5,6-indolequinone. For example, when assaying for the tyrosine hydroxylase activity of

35   tyrosinase, non-tyrosine or altered tyrosine substrates of tyrosinase can be used in addition to tyrosine. In one aspect, melanogenic cells are grown or incubated in culture with a test compound. After pretreatment of the medium, a non-tyrosine or altered tyrosine substrate of

-11-

tyrosinase is added to the growth or incubation medium. The substrate can be a homolog, analog, or derivative of tyrosine which can be a natural product or produced synthetically. Tyrosinase activity in the medium converts the substrate into its product.

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An assay is then used to detect the presence of the product and/or the absence of the substrate. One non-limiting assay is a colorimetric assay. In methods of screening for compounds that inhibit melanogenesis that use a colorimetric assay, a substrate is chosen that changes color when it is acted upon by tyrosinase. That is, the wavelength of light absorbed by the substrate is different than the wavelength of light absorbed by the products of the reaction catalyzed by tyrosinase. The wavelength of light absorbed by the substrate

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and/or by the products can be in the visible light range, the infrared range, or the ultraviolet range of the spectrum. The concentration of substrate, incubation time, and other reaction conditions can be chosen such that the speed and/or intensity of the color change is proportional to the amount of tyrosinase activity in the cells' growth or incubation medium. The color change can be detected by direct observation, or measured by a device, such as a

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spectrophotometer and compared, e.g., to a standard curve prepared using varying amounts of product.

An example of a colorimetric assay is the DOPA oxidase assay. In one method of screening for compounds that inhibit melanogenesis using this assay, a compound to be tested for its ability to inhibit melanogenesis is added to the growth or incubation medium of

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melanogenic cells. After filtration, centrifugation and/or dialyzation of the medium, L-DOPA is added under conditions that would otherwise allow for tyrosinase to catalyze the formation of dopachrome from L-DOPA. In a preferred though non-limiting embodiment, the final concentration of L-DOPA in the medium is about 5×10^{-3} M, the pH is about 7.4, and the temperature is about 25°C. Increased absorbance of the medium at 475 nm (relative to the

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absorbance at 475 nm of medium from similar cells grown under similar conditions but without the test compound) indicates the formation of dopachrome by tyrosinase in the medium, and therefore the inhibition of melanogenesis by the test compound.

Alternatively, as dopachrome absorbs light within the visible range, the presence of dopachrome, and hence the inhibition of melanogenesis, can be determined by direct

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inspection of the reaction, without the aid of a spectrophotometer.

Another assay is a radiometric assay. In an alternative method of screening for compounds that inhibit melanogenesis using this assay, substrate is radioactively labeled and added to the growth or incubation medium to be assayed. If tyrosinase is present in the medium, it cleaves the substrate into a labeled product and an unlabeled product. The

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amount of radioactive substrate that has been converted into radioactive product is measured. The concentration of substrate, time of incubation, temperature of incubation, and other reaction conditions can be chosen so that the amount of radioactive product produced is

-12-

proportional to the amount of tyrosinase in the growth or incubation medium being assayed. A greater amount of labeled product in the medium from cells treated with the test compound than in the medium of similar cells grown under similar conditions but without the test compound indicates that the test compound is a candidate for a compound that inhibits

5   melanogenesis.

An example of this type of assay is the radiometric tyrosine hydroxylase assay. In this assay, the amount of $[^3H]H_2O$ released from $[^3H]$tyrosine as a result of the tyrosine hydroxylase activity of the tyrosinase enzyme is measured. In one method of screening for compounds that inhibit melanogenesis that uses this assay, media from melanogenic cells is

10   harvested and cells removed. Additionally, the media can be dialyzed before assay. For assays, 1.5 microCi $[^3H]$tyrosine is added to the media and incubated for defined lengths of time at appropriate temperature for enzyme activity. Unreacted $[^3H]$tyrosine is removed from the medium by adsorption onto 10% (w/v) activated charcoal in 0.1 M citric acid, then treated with 50% (w/v) Dowex resin solution. The medium is mixed with scintillant and counted in a

15   beta-counter. A significant increase in $[^3H]H_2O$ levels in the medium of cells that were treated with a test compound compared to $[^3H]H_2O$ levels in the medium of similar cells grown under similar conditions without test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

Yet another example of this type of assay is the radiometric melanin synthesis assay.

20   In this assay, the amount of $[^{14}C]$tyrosine or $[^{14}C]$DOPA incorporated into $[^{14}C]$melanin is measured. In a non-limiting example of a method of screening for compounds that inhibit melanogenesis that uses this assay, melanogenic cells are grown or incubated in medium containing a test compound. The medium is harvested and 1 microCi $[^{14}C]$tyrosine is added and incubated at the appropriate temperature for four hours. The reaction is terminated with

25   ice-cold 10% (w/v) TCA and the mixture vortexed and frozen for 24 hours. The mixture is then thawed and centrifuged at 1000 g for 15 minutes at 4°C. The pellet is resuspended in ice-cold 5% TCA (w/v). This step is repeated twice. The final pellet containing $[^{14}C]$melanin is solubilized in Soluene®-350 (Packard Instrument Company, Meriden, CT) for four hours, mixed with scintillant, and counted. Alternatively, the pellet can be collected on filter paper

30   and counted. A significant increase in $[^{14}C]$melanin levels in media of cells that were treated with a test compound compared to $[^{14}C]$melanin levels in media of similar cells grown under similar conditions but without the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

Another assay is a fluorescence assay. In this assay, the substrate and/or its

35   products are fluorescent. The wavelength of light absorbed and/or emitted by the substrate is different from the wavelength of light absorbed and/or emitted by the products. In a non-limiting example of a method of screening for compounds that inhibit melanogenesis that uses

this assay, melanogenic cells are grown in culture in the presence or absence of the test compound. After a period of growth or incubation, the media is removed, tyrosinase substrate added, and a fluorometer used to measure the fluorescence of the growth or incubation medium. The concentration of substrate, time of incubation, temperature of incubation and
5   other reaction conditions can be chosen so that the change in fluorescence is proportional to the levels of tyrosinase activity in the medium being analyzed. A significant difference in fluorescence levels of media from cells treated with a test compound compared to fluorescence levels of media from similar cells grown under similar conditions but without the test compound, indicates that the test compound is a candidate for a compound that inhibits
10   melanogenesis.

Another type of assay involves the precipitation of reaction products. In an example of a method of screening for compounds that inhibit melanogenesis that uses this assay, a substrate of tyrosinase is incubated with the harvested growth or incubation medium under conditions that promote the activity of tyrosinase. The substrate is acted upon by tyrosinase
15   to produce a reaction product that can be precipitated. The reaction product is precipitated. The reaction product can be precipitated from the medium by, for example, increasing or decreasing the temperature of the medium, increasing or decreasing the pH of the medium, increasing or decreasing the ionic strength or salt concentration of the medium, or otherwise altering the medium appropriately, or by centrifugation if the reaction product is insoluble.
20   Substrate concentrations, time of incubation, temperature of incubation, and other reaction conditions can be chosen so that the amount of precipitable reaction product is proportional to the levels of tyrosinase activity in the medium being analyzed. A significant increase in the amount of reaction product precipitated from the media of cells treated with a test compound compared to the amount of reaction product precipitated from the media of similar cells grown
25   under similar conditions but without the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

5.1.1.2   Methods of Screening for Inhibitors of Melanogenesis
Using Assays for Tyrosinase Protein

The preceding methods of screening serve to identify inhibitors of melanogenesis
30   using assays of tyrosinase activity (i.e., the protein's enzymatic activities). The present invention further provides a method of screening inhibitors of melanogenesis using assays for either extracellular or intracellular tyrosinase protein levels. As explained above, tyrosinase is primarily localized to the melanosomal membrane in melanogenic cells. Compounds that cause tyrosinase to be mislocalized serve to inhibit melanogenesis. In the following methods
35   of screening, assays for determining tyrosinase protein levels and/or locations are used. This can be done using any of the standard techniques of protein detection known in the art. The protein detection assays employed herein can be those described in Harlow and Lane

-14-

(Harlow, E. and Lane, D., 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is incorporated herein by reference in its entirety. These assays include, but are not limited to, immunological assays, including Western blots, solid-phase radioimmunoassays, *in situ* hybridizations, and
5   immunoprecipitations. Anti-tyrosinase antibodies are known in the art, and novel anti-tyrosinase antibodies can be generated using well-known techniques. *Id.*

In a non-limiting method of screening for compounds that inhibit melanogenesis, melanogenic cells are grown or incubated in medium containing a test compound. The presence, concentration, or amount of tyrosinase in the medium is determined using a protein
10   detection assay as described above. Test compounds that cause treated cells to secrete more tyrosinase than similar cells grown or incubated under similar conditions but without the test compound are candidates for compounds that inhibit melanogenesis.

Another type of assay that can be used in this screen determines the cellular localization of tyrosinase protein. In wild-type melanogenic cells, most tyrosinase is targeted
15   to the melanosomal membrane, while some tyrosinase is secreted. Mutations or compounds that inhibit melanogenesis (*e.g.*, mutations or compounds that inhibit P protein function) can cause tyrosinase to be secreted to the medium in greater amounts or to be mislocalized to non-melanosomal vesicles. These non-melanosomal vesicles can be separated from melanosomes using subcellular fractionation techniques. In a non-limiting example of a
20   method of screening for compounds that inhibit melanogenesis that uses this assay, melanogenic cells are grown or incubated in medium containing a test compound and the cells are harvested. The subcellular distribution of tyrosinase is then determined in these cells and compared to the subcellular distribution of tyrosinase in similar cells grown or incubated under similar conditions but without the test compound. The assay can incorporate
25   any technique or combination of techniques known in the art, including subcellular fractionation (for example, by sucrose or Percoll density gradient centrifugation), Western blotting of the cells' contents, and tyrosinase activity assays of each subcellular fraction. A decrease in the fraction of total tyrosinase protein found in the melanosomal fraction, or an increase in the fraction of total tyrosinase protein found in a non-melanosomal fraction, in cells
30   treated with the test compound relative to cells not treated with the test compound indicates that the test compound inhibits melanogenesis.

Other qualitative assays can be used, such as, *e.g.*, microscopic examination of cells treated with the test compound. For example, cell staining techniques, as known in the art, can be used. Cells are grown or incubated in medium containing tyrosine and in the presence
35   of a test compound. The cells are stained using anti-tyrosinase antibodies, then examined microscopically. In a non-limiting example of a method of screening using this type of assay, melanogenic cells are grown or incubated in medium containing a test compound, and

-15-

prepared for cell staining using techniques commonly known in the art. *See, e.g.,* Harlow and Lane, 1988, above. Prepared cells are stained using anti-tyrosinase antibodies. The anti-tyrosinase antibodies can be conjugated to a moiety allowing for its detection. Preferably, a secondary antibody is used. The secondary antibody recognizes and binds to the

5   anti-tyrosinase antibody. Preferably, the secondary antibody is conjugated to a moiety allowing for its detection. Alternatively, a tertiary antibody can also be used. The tertiary antibody is preferably conjugated to a moiety allowing for its detection. Examples of moieties allowing for the detection of antibodies include fluorescent molecules (for example, fluoroscein, rhodamine, Hoechst 33258, or Texas red), enzymes (for example, horseradish

10   peroxidase, alkaline phosphatase, or beta-galactosidase), gold particles, radioactive isotope, and biotin. An assay is selected based on the labeling moiety used. For example, fluorescence microscopy can be used to detect fluorescently labeled antibodies. For cells stained with enzyme-conjugated antibodies, the cells are further treated with an appropriate substrate for conversion by the antibody-bound enzyme, followed by examination by light

15   microscopy. Gold-particle labeled antibodies can be detected using light or electron microscopy. Isotope-labeled antibodies can be detected using radiation-sensitive film. For cells stained with biotin-conjugated antibodies, the cells are further treated with streptavidin or avidin. The streptavidin or avidin is conjugated to a moiety that allows for detection such as, for example, a fluorescent molecule, an enzyme, gold particles, or radioactive isotope.

20   Preferably, the cells are co-stained with an antibody or antibodies specific for particular subcellular compartments (*e.g.,* endosomes, lysosomes, melanosomes, etc.). Using any one of these techniques, or any other known technique for detecting antibodies in antibody-stained cells, the subcellular distribution of tyrosinase can be determined. If the test compound causes an increased amount of tyrosinase to be found in non-melanosomal

25   vesicles, and less tyrosinase in melanosomes, then it inhibits melanogenesis.

Another type of assay that can be used determines the presence or absence of the C-terminal portion of the tyrosinase protein. This assay depends, in part, on the discovery that melanogenic cells inhibited for melanogenesis (*e.g.,* by mutations or compounds that inhibit P protein function) contain and secrete a version of tyrosinase that lacks the C-terminal

30   portion of tyrosinase, including its transmembrane domain and its protein sorting signal. As explained above, this truncated form of tyrosinase nonetheless retains catalytic activity. In a non-limiting example of a method of screening based on this assay, melanogenic cells are grown or incubated in the presence of a test compound. An assay is selected that allows the length and/or mass of tyrosinase protein to be determined. For example, Western blots or

35   other immunohistochemical techniques using antibodies that recognize the N-terminal or central portions of the tyrosinase protein, or other standard molecular biological techniques useful for the determination of protein length or mass, can be performed on extracts of these

-16-

cells and/or on their growth or incubation medium. Antibodies appropriate for these assays can be prepared using standard immunological techniques. *See, e.g.*, Harlow and Lane, 1988, above. If the assay reveals the presence of a shorter or lower molecular weight form of tyrosinase, relative to similar cells grown or incubated under similar conditions but without the

5   test compound, then the test compound inhibits melanogenesis. Alternatively, Western blots or other immunohistochemical techniques using antibodies recognizing the C-terminal portion of tyrosinase (*e.g.*, the anti-PEP7 antibody prepared as described in Jimenez *et al.*, 1991, J. Biol. Chem. 266:1147-1156) can be used in the assay. In these assays, a reduction in the amount of tyrosinase protein detected by the antibodies indicates that the test compound

10   inhibits melanogenesis, because the truncated tyrosinase lacks the sequences recognized by the antibodies.

Both full length tyrosinase, and the truncated tyrosinase found in and secreted by melanogenic cells with inhibited or absent P protein, remain catalytically active when run on non-denaturing polyacrylamide gels. This observation is the basis, in part, of another assay

15   for the truncated tyrosinase protein. Thus, melanogenic cells can be grown or incubated in medium containing a compound to be tested. Either the growth or incubation medium is collected, or cell extracts are prepared, and subjected to non-denaturing polyacrylamide gel electrophoresis. Smaller, more flexible proteins will migrate farther than larger proteins with more complicated three-dimensional structure.   Filter paper or a membrane (*e.g.*,

20   nitrocellulose) is soaked in L-DOPA and applied to the gel. Active tyrosinase in the gel converts L-DOPA into melanin, creating dark spots on the filter or membrane indicating the location, and therefore the relative size, of tyrosinase. If cells treated with the test compound produce two spots on the filter or membrane, wherein one spot indicates tyrosinase of the same size as produced by similar cells grown under similar conditions but without the test

25   compound, and the other spot indicates tyrosinase of smaller relative size, then the test compound is a candidate for a compound that inhibits melanogenesis.

Full length tyrosinase in wild-type melanogenic cells with normal P protein function is found primarily in the insoluble fraction of cell extracts. To be released, it must be solubilized with a detergent (*e.g.*, Triton X-100™). In contrast, the smaller truncated version of tyrosinase

30   in melanogenic cells with inhibited P protein function is found in vesicles in the soluble fraction. These observations are the basis, in part, of another assay that can be used to detect truncated tyrosinase in P protein-compromised cells. Thus, melanogenic cells are grown or incubated in medium containing a compound to be tested. The cells are harvested and can be subjected to a detergent phase separation to separate membrane-anchored

35   proteins from soluble proteins. For example, the cells can be solubilized on ice in a buffer containing Triton X-114™. Insoluble contaminants can be spun out at 4°C. Then the supernatant, which contains solubilized proteins, is phase- separated at room temperature or

-17-

elevated temperatures into a detergent phase and an aqueous phase. The ratio of tyrosinase in the detergent phase (which will contain tyrosinase proteins containing the C-terminal portion of the protein which anchors tyrosinase in the membrane) to tyrosinase in the aqueous phase (which will contain tyrosinase proteins which lack the C-terminal portion) is determined.

5   Alternatively, cells are harvested and membranes disrupted by a freeze/thaw cycle or cycles. The disrupted cells are then separated into a soluble fraction and a membrane-bound, insoluble fraction. The ratio of soluble tyrosinase in the soluble fraction to insoluble, membrane-bound tyrosinase in the membrane fraction is determined. If cells treated with the test compound have higher levels of soluble tyrosinase than insoluble, membrane-bound

10   tyrosinase than that from similar cells grown under similar conditions but without the test compound, then the test compound is a candidate for a compound that inhibits melanogenesis.

                    5.1.1.3   Other Methods of Screening for Inhibitors of
                                   Melanogenesis

15   As described above, the mislocalization and secretion of tyrosinase, and the reduction of tyrosinase activity, are not the only results of an inhibition of melanogenesis. Other melanogenic enzymes are also affected, as is the biogenesis of melanosomes.

Inhibition of melanogenesis by mutations that inhibit P protein function can cause a marked alteration in the amount of several melanogenic proteins produced in melanocytes,

20   including the TRP-1, TRP-2, and LAMP-1 gene products (Orlow, S.J., and Brilliant, M.H., 1999, above). In the eyes of wild-type mice, the levels of TRP-1 and TRP-2 gene products are high at birth, fall sharply, increase gradually to another peak at about 2 weeks, then permanently fall to undetectable levels by about 40 days. In mice that lack P protein function, for example, the levels of these proteins are much lower at birth and are undetectable after

25   only a few days (id.).

Another effect of inhibited melanogenesis caused by a mutation which inhibits P protein function is the disruption of a high molecular weight complex comprising tyrosinase, TRP-1 protein, and TRP-2 protein (Orlow, S.J. et al., 1994, above). For purposes of the present invention, the term "high molecular weight complex" is defined as a group of proteins

30   bound to each other via covalent and/or non-covalent bonds that remain associated with each other during non-denaturing gel filtration, HPLC, or sucrose gradient sedimentation and have an apparent molecular weight of between about 200 kD and about 700 kD. In wild-type melanogenic cells, this "melanogenic complex," which is associated with the melanosome, contains a significant fraction of the cells' complement of tyrosinase, TRP-1 protein and

35   TRP-2 protein. In melanogenic cells inhibited for melanogenesis by inhibition of P protein function, very little of any of these proteins is found in high molecular weight complexes.

Another assay takes advantage, in part, of these effects. In a non-limiting example of a method of screening for compounds that inhibit melanogenesis that uses this type of assay, melanogenic cells are grown or incubated in medium containing a compound to be tested. The cells are harvested, disrupted, and fractionated. This fractionation can be done, for

5   example, using sucrose gradient sedimentation. Aliquots from the sedimentation are assayed for the presence of melanogenic proteins such as, for example, by using assays for tyrosinase, TRP-1, and/or TRP-2 activity, or by using immunohistochemical assays, such as immunoblotting. Increased amounts of any of these three proteins in low density aliquots and/or decreased amounts of any of these three proteins in high density aliquots, relative to

10   similar cells grown or incubated under similar conditions but without the test compound, indicate that the test compound is a candidate for a compound that inhibits melanogenesis.

Another consequence of inhibited melanogenesis can be the aberrant development of melanosomes. Wild-type melanogenic cells typically contain abundant, fully developed, darkly pigmented melanosomes. Such fully developed, darkly pigmented melanosomes are

15   less abundant or absent in melanocytes inhibited for melanogenesis due to a mutation in the P protein-encoding gene when they are grown or incubated in medium containing low concentrations of tyrosine. Rather, these cells contain an unusually large number of immature melanosomes. This phenomenon is the basis, in part, for another assay that can be used. In a non-limiting example of a method of screening for compounds that inhibit

20   melanogenesis that uses this type of assay, melanogenic cells are grown or incubated in medium containing a test compound. The number, size, shape, and/or color of the melanosomes in the cells is assayed. Such assays are well known in the art. For example, cells can be fixed and stained and examined using light microscopy. Alternatively, cells can be fixed, stained, sectioned, and examined using electron microscopy. Alternatively, cells can

25   be fractionated using density centrifugation. Mature melanosomes are denser than immature melanosomes, and so can be separated from them on the basis of density using well known techniques. Cells treated with a test compound that have melanosomes that are altered in number, size, shape, and/or color compared to melanosomes from similar cells grown or incubated under similar conditions but without the test compound indicates that the test

30   compound inhibits melanogenesis.

5.1.1.4   Methods of Screening for Inhibitors of P Protein
              Function

As explained above, the P protein plays a pivotal role in melanogenesis. Melanocytes with loss of function mutations in the P protein-encoding gene are inhibited for

35   melanogenesis. In P deficient or P inhibited cells, inhibition of melanogenesis is correlated with mislocalization of tyrosinase. Whereas in wild-type melanocytes tyrosinase is localized primarily to melanosomes, in melanocytes with loss of function mutations in the P

-19-

protein-encoding gene, tyrosinase is predominantly secreted or found in non-melanosomal vesicles. Inhibition of melanogenesis and the mislocalization of tyrosinase can be mimicked by treating wild-type melanocytes with compounds that inhibit the function of P protein (e.g., imipramine).

5   These discoveries are the basis, in part, for a number of screens for inhibitors of melanogenesis. These screens serve to identify inhibitors of melanogenesis by identifying inhibitors of P protein function. Thus, melanogenic cells are grown or incubated in medium containing a compound to be tested for its ability to inhibit P protein function. The effect, if any, of the compound can be determined using, for example, any one of the assays described 10   above. In a non-limiting embodiment, the activity of tyrosinase in the growth or incubation medium of the cells can be measured. For example, tyrosine can be added to the medium, and its conversion to melanin monitored. Alternatively, non-tyrosine or altered tyrosine substrates of tyrosinase can be added to the medium, and their conversion into reaction products by tyrosinase can be followed by, for example, colorimetric assays (e.g., the DOPA 15   oxidase assay), radiometric assays (e.g., the radiometric hydroxylase or radiometric melanin synthesis assays), fluorescence assays, or by the precipitation of reaction products. These assays are described in detail in Section 5.1.1.1, above.

Alternatively, the assays of tyrosinase protein may be used. These assays can measure, for example, the amount of tyrosinase in the growth or incubation medium of the 20   cells treated with the compound to be tested, the cellular localization of tyrosinase (e.g., by subcellular fractionation of the cells, or by staining and microscopic examination of the cells), or the length or mass of the tyrosinase molecules within the cells. These assays are described in detail in Section 5.1.1.2, above.

Other assays that can be used include those that measure other effects of the 25   inhibition of P protein function. For example, these assays can measure the amount or activity of TRP-1 and/or TRP-2 protein in cells treated with the compound to be tested, the abundance or composition of the high molecular weight melanogenic complex, or the presence or absence of aberrant melanosomes. These assays are described in detail in Section 5.1.1.3, above.

30   Still another assay that can be used involves measuring the intracellular targeting, intracellular levels and/or secretion of a certain class of lysosomal hydrolases. Normally, newly synthesized lysosomal hydrolases are transported from the trans-Golgi network to a late endosome compartment, portions of which are thought to eventually fuse with or form lysosomes. These lysosomes, containing most of the intercellular lysosomal hydrolase 35   activity, can be detected in a large granule fraction prepared from fractionated cells. As illustrated below by way of a non-limiting example, some, but not all, lysosomal hydrolases are not correctly targeted to the lysosome-containing large granule fraction from melan-p cells

-20-

as opposed to melan-a cells. In particular, lysosomal hydrolases that are transported from the
trans-Golgi network to the late endosome via binding to the mannose-6-phosphate/insulin-like
growth factor type II receptor (the "M6P/IGF-II receptor") do not accumulate in the large
granule fraction. Such incorrectly targeted lysosomal hydrolases include β-hexosaminidase,
5   β-glucosidase, β-glucuronidase and β-galactosidase. In contrast, acid phosphatase, which is
not transported to the late endosome via the M6P/IGF-II receptor, correctly accumulates in
the large granule fraction in both melan-a and melan-p cells. Thus, P protein function is also
necessary for the correct targeting of lysosomal enzymes that are transported to the late
endosome via the M6P/IGF-II receptor. The default pathway for such enzymes is secretion
10   into the exterior of the cell.

These results are the basis, in part, for additional methods of screening for
compounds that affect P protein function. Accordingly, in lieu of, or in addition to, assays for
the mislocalization of tyrosinase, one can screen for the effect of a test compound on the level
and/or localization of any lysosomal hydrolase that is normally transported to the late
15   endosome via the M6P/IGF-II receptor, including but not limited to β-hexosaminidase, β-
glucosidase, β-glucuronidase and β-galactosidase. Since these lysosomal hydrolases are,
like tyrosinase, proteins and more particularly enzymes, any of the methods described above
to assay for the presence of tyrosinase's enzymatic activity and/or protein can be adapted to
assay lysosomal hydrolases. Assays for the enzymatic activity of these enzymes are well
20   known in the art (and, in part, illustrated below by way of non-limiting example), as are their
amino acid structures and antibodies that recognize the same. For example, one can assay
for the presence and/or levels of lysosomal hydrolases in whole cells or cell extracts, in the
large granule fraction of a cell extract, and/or in the medium from cells treated with test
compounds. Compounds that cause either a decrease in accumulation of such lysosomal
25   enzymes in cells or, more particularly, the large granule fraction, or an increase in secretion of
such lysosomal enzymes, are candidates for compounds that inhibit the function of P protein;
such candidate compounds are then further analyzed using one of the other methods of the
invention.

5.1.2   Methods of Screening for Compounds that Increase Melanogenesis,
30   Increase P Protein Function and/or Mimic P Protein Function

As explained above, wild-type melanogenic cells typically secrete a portion of their
tyrosinase into *in vitro* culture medium. Although the secreted tyrosinase is enzymatically
active, it cannot contribute to melanogenesis, which occurs inside the cells' melanosomes.
As described above, the level of melanogenesis within melanogenic cells is proportional to the
35   fraction of tyrosinase that is localized to melanosomes. Compounds that decrease the
amount of tyrosinase localized to melanosomes serve to inhibit melanogenesis. Conversely,
compounds that reduce the amount of tyrosinase that is secreted, and thereby increase the

-21-

amount of tyrosinase localized to melanosomes, are expected to increase melanogenesis. As explained above, P protein activity is required for the localization of tyrosinase to melanosomes. Thus, compounds that increase the activity of P protein in melanogenic cells, as well as compounds that mimic the activity of P protein, will increase melanogenesis by
5   reducing the amount of tyrosinase that is secreted.

A number of screens based, in part, on these observations and predictions can be used to identify compounds that increase melanogenesis, increase the function of P protein, and/or mimic the function of P protein. For example, variations of the assays described above using melanogenic cells to identify inhibitors of melanogenesis and P protein function can be
10 used. Melanogenic cells are grown or incubated *in vitro* in medium containing a compound to be tested for its ability to increase melanogenesis, increase P protein function or mimic P protein function. The effect, if any, of the compound can be determined using, for example, any one of the assays described above. For example, the activity of tyrosinase in the growth or incubation medium of the cells can be measured. A decrease in the activity of tyrosinase in
15 the medium may indicate that less tyrosinase is being secreted, and that the compound might therefore increase melanogenesis or P protein function.

Alternatively, or in addition, melanogenic cells that do not contain P protein (*e.g.*, melan-p cells) can be used to screen for compounds that mimic P protein function. In one type of assay that can be used in the invention, melanogenic cells that do not contain P
20 protein are incubated in medium containing a compound to be tested for its ability to mimic P protein function and increase melanogenesis. In contrast to normal melanogenic cells, such melanogenic cells that do not contain P protein are light colored in culture (as well as in the animal). If the melanogenic cells that do not contain P protein turn darker in the presence of the compound than in the absence of the compound, then the compound mimics P protein
25 function in whole or in part. The color of the cells can be measured qualitatively such as, for example, by visual inspection, or quantitatively, such as, for example, by reflectance. Alternatively, melanogenic cells that do not contain P protein are treated with the compound to be tested, and the amount of tyrosinase secreted into the medium is assayed. If the amount of tyrosinase in the medium from melanogenic cells that do not contain P protein
30 (*e.g.*, melan-p cells) decreases when the cells are treated with the test compound, then the test compound is a candidate for a compound that mimics P protein function. Tyrosinase activity in the medium can be measured, for example, by using any of the techniques described above. For example, tyrosine can be added to the medium, and its conversion to melanin monitored.

35   Alternatively, assays of tyrosinase protein may be used. These assays can measure, for example, the amount of tyrosinase in the growth or incubation medium of the cells treated with the compound to be tested, the cellular localization of tyrosinase (*e.g.*, by subcellular

-22-

fractionation of the cells, or by staining and microscopic examination of the cells), or the length or mass of the tyrosinase molecules present within the cells. A decrease in the amount of tyrosinase protein secreted into the medium, or an increase of tyrosinase protein in melanosomes, indicates that the test compound is a candidate for a compound that increases

5   melanogenesis or mimics or enhances P protein function. If the test compound causes similar effects in melanogenic cells that do not contain P protein (e.g., melan-p cells), such a result would indicate that the compound mimics P protein function. These assays are described in detail in Section 5.1.1.2, above.

Other assays that can be used include those that measure other effects of an

10   increase in P protein function, mimic of P protein function, and/or an increase in melanogenesis. For example, these assays can measure the amount of TRP-1 and/or TRP-2 protein or activity in cells treated with the compound to be tested, the abundance or composition of the high molecular weight melanogenic complex, or the presence or absence of aberrant melanosomes as described above. An increase in the amount of TRP-1 and/or

15   TRP-2 protein or activity, an increase in the amount of these proteins found in high molecular weight melanogenic complexes, or an increase in the number of high molecular weight complexes, indicates that the test compound is a candidate for a compound that increases melanogenesis or P protein function. If the test compound causes similar effects in melanogenic cells that do not contain P protein (e.g., melan-p cells), such a result would

20   indicate that the compound mimics P protein function. These assays are described in detail in Section 5.1.1.3, above.

In a variation of these screens, the amount of secreted tyrosinase is compared to the amount of intracellular tyrosinase. Melanogenic cells are grown or incubated in medium containing a compound to be tested. Using, for example, any of the assays described above,

25   the amount of tyrosinase in the growth or incubation medium is determined and the amount of tyrosinase within these cells is also determined. The ratio of intracellular tyrosinase to secreted tyrosinase is then calculated. If this ratio is higher for cells treated with the compound to be tested than for similar cells grown under similar conditions but without the compound, then the compound increases melanogenesis. In a non-limiting preferred

30   embodiment, such a change in the ratio of intracellular tyrosinase to secreted tyrosinase is observed without a change (e.g., reduction) in the total amount of tyrosinase produced by the cell. Similarly, if melanogenic cells that do not contain P protein (e.g., melan-p cells) are grown or incubated in medium containing the compound to be tested, and the ratio of intracellular tyrosinase to secreted tyrosinase is higher for cells treated with the compound

35   than for untreated cells, then the compound can mimic P protein function, and thereby increase melanogenesis.

-23-

5.1.3   Methods of Screening for Compounds That Affect P Protein Function Using Non-Melanogenic Cells

Most non-melanogenic cells do not express P protein or tyrosinase. For purposes of the present invention, the term "non-melanogenic cells" is defined as cells that do not contain

5   melanosomes. However, non-melanogenic cells can be made to express both P protein and tyrosinase, and to synthesize melanin. For purposes of the present invention, the term "cells made to express both P protein and tyrosinase" is defined as cells that do not ordinarily express P protein and/or tyrosinase, but are caused to express both P protein and tyrosinase using any technique known in the art such as, e.g., molecular genetic techniques. For

10   example, heterologous tyrosinase and/or P protein genes can be introduced into the cells by, e.g., transfection, transformation, or transduction. For purposes of the present invention, the term "heterologous" is defined as describing a gene or gene product that does not naturally exist in that organism, or a gene or gene product that is not normally expressed in that cell type. Alternatively, endogenous, but normally quiescent, tyrosinase and/or P protein-

15   encoding genes can be activated to express tyrosinase and/or P protein (e.g. through targeted homologous recombination of transcriptional control sequences, or any other activation method). Several methods of the present invention are based, in part, on the discovery that non-melanogenic cells expressing P protein and tyrosinase together have almost four times as much tyrosinase activity as cells expressing tyrosinase alone. Cells

20   expressing P protein, but not tyrosinase, do not have detectable tyrosinase activity, showing that P protein's effect on tyrosinase activity in these cells is completely dependent on the expression of tyrosinase.

The tyrosinase activity of cells made to express both tyrosinase and P protein is sensitive to the action of compounds that inhibit P protein function. When these cells are

25   treated with, for example, imipramine, the tyrosinase activity of these cells is markedly reduced. The effect of these compounds on tyrosinase activity is totally dependent on the presence of active P protein. Cells expressing tyrosinase but not P protein have tyrosinase activities that are unaffected by the presence of the compound at the concentrations tested.

These observations are exploited in a number of methods of screening for

30   compounds that affect (e.g., decrease or increase) P protein function. Cells that do not otherwise have detectable tyrosinase and/or P protein are made to express both of these proteins. The cells are grown or incubated in medium that contains a compound to be tested. The tyrosinase activity of extracts of these cells is measured. Tyrosinase activity can be measured using any of the assays discussed above, including the radiometric tyrosine

35   hydroxylase assay, colorimetric DOPA oxidase assay, the DHICA converting assay, an assay for the ability to convert $[^{14}C]DOPA$ into TCA precipitable material, or by any other method known in the art. If the tyrosinase activity of the extracts of cells treated with the test

compound is lower than the tyrosinase activity of the extracts of similar cells grown under similar conditions but without the test compound, and if the compound does not otherwise decrease tyrosinase activity in the extracts of cells expressing tyrosinase but not P protein, then the compound decreases P protein function. Conversely, if the tyrosinase activity of the

5   extracts of cells treated with the test compound is higher than the tyrosinase activity of the extracts of similar cells grown under similar conditions but without the test compound, and if the compound does not otherwise increase tyrosinase activity in the extracts of cells expressing tyrosinase but not P protein, then the compound increases P protein function.

Another method of screening using non-melanogenic cells made to express

10   tyrosinase and P protein exploits, in part, the discovery that these cells, if incubated long enough, turn black with melanin deposition. Cells expressing tyrosinase and P protein, or tyrosinase but not P protein, are treated with a compound to be tested. The cells are incubated for a period of time sufficient to allow cells expressing both tyrosinase and P protein, but which are not treated with the test compound, to accumulate melanin. The

15   melanin content of treated and untreated cells can be assayed by visual inspection or spectrophotometric analysis of the cells, or by using other techniques well known in the art. If the melanin content of the cells expressing both tyrosinase and P protein and treated with the test compound is lower than the melanin content of similar cells not treated with the compound, then the compound can decrease melanogenesis. If the melanin content of cells

20   expressing tyrosinase but not P protein is not substantially altered by the presence or absence of the compound, then the compound inhibits P protein function. Conversely, compounds that cause an increase in melanin formation in these cells, relative to similar cells grown under similar conditions but without the compound, increase melanogenesis. If the compound also fails to increase melanin formation in non-melanogenic cells expressing a

25   tyrosinase-encoding gene but not a P protein-encoding gene, then the compound increases P protein function.

Alternatively, broken cell extract systems can be devised to study intracellular trafficking of tyrosinase. In a non-limiting example, donor Golgi membranes and cytosol from wild-type melanocytes can be combined with melanosomes prepared from cells of a mouse

30   with a mutation in the tyrosinase gene that inactivates the enzyme. One could then observe the transfer of tyrosinase from the wild-type donor Golgi membranes to the tyrosinase-deficient melanosomes. Addition of a compound that inhibits P protein function would inhibit such transfer.

For those methods using heterologous genes, the heterologous tyrosinase and P

35   protein-encoding genes can be derived from any suitable source. Preferably, they are derived from an animal source. More preferably, they are derived from a mammalian source such as the mouse cells used below in illustrating embodiments. Even more preferably, they are

-25-

derived from a primate source such as humans. Tyrosinase-encoding genes are well known in the art (see, for example, expression of the human gene cDNA in Bouchard *et al.*, 1989, J. Exp. Med. 169 (6), 2029-2042, and the MEDLINE database at accession nos., for example, NM_000372, M27160, and U01873), as are P protein-encoding genes (see for example, 5   Rinchik *et al.*, 1993, Nature 361 (6407), 72-76, and the MEDLINE database at accession nos., for example, NM_000275 and U19152 for the human gene).

Expression cassettes are typically used to express heterologous genes in the chosen cell. Each expression cassette contains regulatory sequences designed to express, for example, the tyrosinase-encoding gene and/or the P protein-encoding gene. For expression 10   in prokaryotic cells, preferably each coding sequence found in the expression cassette is operatively linked to at least one regulatory sequence, *i.e.*, a promoter sequence. By "operatively linked" is meant that the regulatory sequence functions to regulate the coding sequence (*e.g.*, controls the timing or amount of expression of the coding sequence, determines initiation or termination of transcription or translation, or affects message stability). 15   For expression in eukaryotic cells, preferably each coding sequence found in the expression cassette is "operatively linked" to at least two regulatory sequences, *i.e.*, a promoter and a polyA sequence. Each expression cassette is operatively linked to the polynucleotide sequence of a vector. Each vector preferably contains polynucleotide sequences that allow for its selection, replication, and maintenance in transfected cells, either as an autonomous 20   extrachromosomal element, or as an integrated component of one or more chromosomes in the transfected cells. Vectors containing expression cassettes that can be adapted to express almost any coding sequence are well known in the art and commercially available. Non-limiting examples of such vectors are illustrated below using the pcDNA vectors available from Invitrogen (San Diego, CA).

25   Any promoter that facilitates a sufficiently high rate of expression can be used in the expression cassette. The promoter can be constitutive or inducible. *See, e.g.*, Resendez et al., 1988, Mol. Cell Biol. 8:4579-4584; and Chang et al., 1987, Proc. Natl. Acad. Sci. USA 84:680-684, which describe inducible promoters. The choice of the promoter depends on what cell type is used in the screen and the desired level of expression of the heterologous 30   genes encoding tyrosinase and/or P protein. *See, e.g.*, Gossen *et al.*, 1995, Science 268:1766-1769; Gossen and Bujard, 1992, Proc. Natl. Acad. Sci. USA 89:5547-5551 and U.S. Patent Nos. 5,851,984; 5,849,997; 5,827,687; 5,811,260; 5,789,215; 5,665,578; 5,512,483; 5,302,517; 4,959,313; and 4,935,352, which describe useful promoter sequences.

Further non-limiting examples of promoter sequences and elements include the SV40 35   early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad.

-26-

Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, *et al.*, 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), and the *tac* promoter (DeBoer, *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful

5   proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella *et al.*, Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, *et al.*, 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella *et al.*, 1984, Nature 310:115-120); promoter elements from

10   yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: the elastase I gene control region, which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant.

15   Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); the insulin gene control region, which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122); the immunoglobulin gene control region, which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444); the mouse mammary tumor virus control region, which is active in

20   testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495); the albumin gene control region, which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276); the alpha-fetoprotein gene control region, which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58); the alpha 1-antitrypsin gene control region, which is active in the liver (Kelsey *et al.*, 1987, Genes and

25   Devel. 1:161-171); the beta-globin gene control region, which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94); the myelin basic protein gene control region, which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); the myosin light chain-2 gene control region, which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); and the gonadotropic releasing

30   hormone gene control region, which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

       Another regulatory element that can be used in the expression cassette for eukaryotic cell expression is a polyA sequence (or polyA signal), which should be capable of efficiently inducing polyadenylation of a transcript specific for the coding sequence to which the polyA

35   sequence is operatively linked. *See, e.g.*, U.S. Patent Nos. 5,861,290; 5,851,984; 5,840,525 and 5,627,033, which discuss polyA sequences.

-27-

In another non-limiting embodiment, the expression cassette used according to the present invention may further comprise an enhancer element, a 5' or 3' untranslated sequence (or region), one or more introns, a sequence that regulates RNA stability, or a combination of more than one of these elements. Any sequence that falls into any of these
5   categories can be used in the vector of the present invention. See U.S. Patent Nos. 5,861,290; 5,851,984; 5,840,525; 5,681,744 and 5,627,033, which discuss these regulatory elements. The term "5' untranslated sequence" refers to the sequence of an mRNA molecule between the transcription initiation site and the translation initiation site. The term "3' untranslated sequence" refers to the sequence of an mRNA molecule between the
10   translation termination site and the polyA tail.

The heterologous genes used in these assays are typically introduced into the chosen cells by transfection, transduction, transformation, or any other suitable technique known in the art. For example, electroporation, calcium phosphate coprecipitation, microinjection, lipofection, etc., can be used. See, e.g., U.S. Patent No. 5,814,618 and 5,789,215, which
15   describe transfection methods. The cells that take up the heterologous gene or genes, either through integration into their genome or by maintenance as part of an extrachromosomal element, are then preferably selected by standard techniques. Thus, a selectable marker can be included in the vector which allows a cell that has the marker, and thus cells that contain the vector and the heterologous gene or genes, to be isolated from cells that do not have the
20   marker. Whether a selectable marker is necessary to prepare the cells used in these assays depends on the particular method by which the vector is introduced into the cells. For example, if the vector is introduced into the cells via microinjection, a selectable marker may be less useful than if electroporation is used because the transformation frequency tends to be higher for microinjection. For example, the marker can enable a cell to grow under
25   selective conditions, i.e. conditions under which the cell could not grow if it did not have the marker (e.g., the neomycin resistance gene and the hypoxanthine phosphoribosyltransferase gene). A marker can also provide another means by which to identify the cell which took up the heterologous polynucleotide molecule or vector (e.g., by preferential staining). See, e.g., U.S. Patent Nos. 5,851,984 and 5,789,215, which describe selectable markers.

30   The cells used in these assays can typically be derived from any source. The cells used in these assays can be cells derived from a mammalian animal, for example: a sheep, cow, pig, or other farm animal; a cat, dog, or other domesticated animal; a mouse, rat, or other rodent; a monkey, ape, or other primate; and most preferably a human. Alternatively, the cells used in these assays can be derived from non-mammalian animals such as, for
35   example, a bird, fish, reptile, amphibian, or insect. Cells derived from animals for use in these assays can be of any type such as, for example, fibroblasts, glial cells, keratinocytes, hepatocytes, ependymal cells, bone marrow cells, hippocampal cells, stem cells, embryonic

stem cells, hematopoietic stem cells, olfactory mucosa cells, adrenal cells, leukocytes, lymphocytes, chromaffin cells, neurons, cells of the immune system, macrophages, Schwann cells, oligodendrocytes, astrocytes, germline cells, somatic cells, epithelial cells, endothelial cells, adrenal medulla cells, osteoblasts, osteoclasts, myoblasts, pancreatic cells (e.g., of the

5   islets of Langerhans), or a mixture of more than one of the above cell types, etc. Alternatively, the cells used in these assays can be derived from a plant source such as, for example, a dicotyledon such as, e.g., tobacco, or a monocotyledon, such as, e.g., corn. Alternatively, the cells used in these assays can be derived from a unicellular eukaryotic organism such as, for example, a protozoan or a yeast or other unicellular fungus. Methods

10   of growing these cells are specific to each cell type and within the skill of the art.

In a preferred embodiment, established cell lines from any of these sources can be used for these assays. Examples of suitable cell lines include, but are not limited to, Chinese Hamster Ovary (CHO) cells, HeLa cells, NRK cells, A293 cells, and COS cells. The cells should have the ability to proliferate when grown in in vitro culture. Following introduction of

15   the heterologous gene or genes into the cells, and selection for cells that have taken up the heterologous gene or genes, such cells, in a preferred embodiment, should be useful to establish a cell line that can be grown, stored, re-grown, etc., for extended periods of time in in vitro culture. See, e.g., U.S. Patent No. 5,814,618, which describes cells useful for the assays of the present invention.

20   5.1.4   High-Throughput Methods of Screening for Compounds that Affect or
                              Mimic P Protein Function

The methods of screening for compounds that affect or mimic P protein function described above can be used to test individual compounds or small numbers or large numbers of compounds contemporaneously. High-throughput methods of screening, as

25   known in the art, are preferable.

For purposes of the present invention, the term "high-throughput method of screening" is defined as a method of screening that allows for large numbers of compounds to be tested concurrently. Each or all of the steps in screening compounds that affect or mimic P protein function are amenable to high throughput methods of screening for candidate

30   compounds. Preferably, the high-throughput methods of screening are partially or fully automated, reducing the amount of attention required to test each compound. For example, an increase in the amount of tyrosinase secreted into the medium, or total levels of tyrosinase activity, can be detected easily in the formats (such as, e.g., 96 well plates) typically used in high-throughput methods of screening. High-throughput methods of screening are well known

35   in the art and can be performed in any of a number of formats. Laboratory automation, including robotics technology, can significantly decrease the time necessary to screen large numbers of compounds, and is commercially available from, for example, Tecan (Research

Triangle Park, NC), Scitec Laboratory Automation SA (Lausanne, Switzerland), Rosys (New Castle, DE), Rixan Associates Inc. (Dayton, OH), CRS Robotics (Burlington, Ontario Canada), Fanuk Robotics, and Beckman-Coulter Sagian (Indianapolis, IN), to name just a few companies. Upon identifying candidate compounds, secondary methods of screening can be

5   performed to determine the cellular and/or *in vivo* effects of the candidate compounds on P protein function.

        5.1.5   Secondary Methods of Screening and Additional Methods of Screening for Compounds That Affect or Mimic P Protein Function

Each of the above methods of screening can be used by itself to identify compounds

10   that are likely to affect or mimic P protein function. Alternatively, a plurality of methods of screening can be used serially to confirm, or to determine more accurately, the P protein affecting properties of one or more compounds. For example, any of the above methods of screening can be used as a primary method of screening, followed by a secondary method of screening. For purposes of the present invention, the term "primary method of screening" is

15   defined as the first method of screening used to test the ability of a compound to affect or mimic P protein function. For purposes of the present invention, the term "secondary method of screening" is defined as any method of screening that is not the primary method of screening. The use of secondary methods of screening is particularly important when the primary method of screening is based on the identification of compounds that lower the

20   activity of tyrosinase or the amount of melanin produced, or that lower the amount of tyrosinase secreted. Direct inhibitors of tyrosinase will also cause a reduction in the activity of tyrosinase and the amount of melanin produced, or can cause a reduction in tyrosinase activity, but would not necessarily affect P protein function.

Any of the methods of screening described above can also be used as a secondary

25   method of screening. For example, one can identify candidate compounds using as a primary screen the assay for an effect on tyrosinase activity in cells made to express tyrosinase and P protein, yet which don't affect tyrosinase activity in cells made to express tyrosinase alone. Promising compounds from this primary screen can then be tested in a secondary screen in an assay for their effect on cellular localization of tyrosinase and/or lysosomal enzymes in

30   melanogenic cells. Of the methods of screening described above, the ones which rely upon identification of the mislocalization of tyrosinase protein or activity or size are preferred as secondary methods of screening.

In one embodiment, a secondary screen is employed to distinguish the effects of test molecules that effect the melanogenic pathway in general and P-protein in particular, and

35   those that inhibit protein synthesis, trafficking and proteolysis. For example, in an assay for activators of P-protein function, a secondary screen can simply entail visually examining the test melanocytes to ensure a darker color and therefore an increase in P-protein activity,

-30-

rather than a general inhibition of protein synthesis, trafficking or proteolysis by the test molecule and resulting decrease in tyrosinase secretion. Alternatively, the cells can be histologically examined, preferably by electron microscopy, optionally together with DOPA staining (as described in Section 9, *infra*), to determine their melanosome content. A true

5   activator of P-protein activity will promote the maturation of melanosomes from stages I-III to stages III-IV, whereas an inhibitor of protein synthesis, trafficking and proteolysis is unlikely to promote melanosome maturation.

Other methods of screening can be used. For example, compounds can first be screened for binding affinity to purified P protein. Alternatively, a compound identified by a

10   primary method of screening as affecting P protein function can be tested for direct binding to purified P protein *in vitro*, or by copurification with P protein from P protein-expressing cells treated with the compound. Each of these methods of screening can determine whether the compound binds directly to P protein. A compound that can bind directly to P protein and which also affects tyrosinase activity or localization or some other aspect of melanogenesis is

15   likely to directly affect P protein function. Alternatively, a compound identified by a primary method of screening as affecting P protein function can be tested for the ability to affect tyrosinase directly. For example, the test compound can be added to a system that contains tyrosinase but not P protein. Such a system can be, for example, an *in vitro* system containing purified or partially purified tyrosinase protein free or essentially free of P protein.

20   Alternatively, it can be a cell that expresses tyrosinase but not P protein. If the effect of the test compound on tyrosinase is P protein independent, then the test compound does not affect P protein function. If the effect of the test compound on tyrosinase is also observed in the absence of cellular trafficking (*e.g.*, on purified tyrosinase protein, and not in cells), then the test compound does not mimic P protein function.

25   While preferred primary methods of screening, especially those that are high-throughput methods of screening, are those with the lowest costs (that is, can be performed as quickly, with as little human supervision, and using as few materials as possible), secondary methods of screening can be more time, labor, and material-intensive. This is because the secondary methods of screening are performed only on test compounds

30   that are identified by the primary method of screening as affecting or mimicking P protein function. These compounds are expected to be a small fraction of the total number of compounds tested in any large scale, high-throughput screening effort. Examples of methods of screening that are better suited for secondary screens than for primary screens include administration of a test compound to an animal (*e.g.*, topically, subcutaneously, or orally) or to

35   animal skin equivalents grown in culture, where lightening of the skin or skin equivalent indicates that the compound inhibits P protein function. Or, for compounds that mimic P protein function, the secondary screen can include administration of the test compound to a

melan-p animal or animal skin equivalent, where darkening of the skin or skin equivalent indicates that the compound mimics P protein function.

Primary and secondary methods of screening can be used in another way to identify compounds that affect or mimic P protein function. Once a compound that affects or mimics

5   P protein function is identified by using, for example, a primary method of screening, chemical analogs of the compound can be selected or created. For purposes of the present invention, the term "chemical analog" is defined as a compound that is chemically related to another chemical compound. The relationship is preferably structural as known in the art such as where, for example, the two compounds differ only in the location of a substituent, such as,

10 e.g., a hydroxyl or alkyl group, or are chemical homologs of each other. Alternatively, the relationship might be functional such as where, for example, both compounds affect the same mechanism, such as, e.g., where both compounds are kinase inhibitors. Methods for designing or selecting chemical analogs are described below in Section 5.2. These chemical analogs can then be tested for the ability to affect or mimic P protein function using, for

15 example, any method described above. The secondary method of screening can be the same as the primary method of screening, or it can be a different method of screening. Chemical analogs are sought which have a stronger effect on P protein function than the original test compound. This procedure can be repeated serially to identify or create compounds of increasing efficacy.

20 5.2   Compounds for Inhibiting, Increasing or Mimicking P Protein Function

Compounds that can be screened in accordance with the present invention include but are not limited to small organic molecules that are able to gain entry into a cell and affect P protein activity. A number of compound libraries are commercially available from companies such as Pharmacopeia (Princeton, NJ), Arqule (Medford, MA), Enzymed (Iowa

25 City, IA), Sigma-Aldrich (St. Louis, MO), Maybridge (Trevillett, United Kingdom), Trega (San Diego, CA) and PanLabs (Bothell, WA), to name just a few sources. One also can screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds that affect or mimic P protein function.

30 One class of preferred compounds for use in the methods of the present invention comprises chemical analogs of imipramine. As described above, imipramine inhibits P protein function. Imipramine is a tricyclic tertiary amine used in the treatment of depression. *See* Gilman, A.G. *et al.*, eds, 1990, *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, Eighth Edition, 405-14, Pergamon Press, New York. Other tricyclic tertiary

35 amines used in the treatment of depression such as, for example, amitriptyline, trimipramine, or doxepin (*see id.*) can be test compounds in screens for compounds that affect P protein function. Secondary amines used in the treatment of depression such as, for example,

-32-

desipramine, nortriptyline, protriptlyine, amoxapine, or maprotiline (see id.) also are preferred compounds for the screens of the present invention. These chemical analogs of imipramine all share structural and functional characteristics with imipramine. Other chemical analogs of imipramine that are preferred compounds for use in the methods of the present invention
5   include chemicals with functional and/or structural similarities to imipramine. For example, the atypical antidepressants such as, for example, trazodone and fluoxetine, lack structural similarity with imipramine (see id.), but share the functional property with imipramine of being useful antidepressants, and so are preferred compounds for the screens of the present invention. Tricyclic compounds, tertiary amines, and secondary amines without
10   antidepressant effects also are preferred compounds of the present invention.

Once a compound that affects or mimics P protein function is identified, molecular modeling techniques can be used to design chemical analogs of the compound that are more effective. For example, chemical analogs of imipramine, or any of the other preferred compounds listed above, can be created using these or other modeling techniques.
15   Examples of molecular modeling systems are the CHARM (Polygen Corporation, Waltham, MA) and QUANTA (Molecular Simulations Inc., San Diego, CA) programs. CHARM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules
20   with each other.

For example, once a compound that affects or mimics P protein function is identified, the compound can be used to generate a hypothesis. Such a hypothesis can be generated from any one of the preferred compounds of the present invention using, e.g., the program Catalyst (Molecular Simulations Inc., San Diego, CA). Furthermore, Catalyst can use the
25   hypothesis to search proprietary databases such as, for example, the Cambridge small molecule database (Cambridge, England), as well as other databases or compound libraries, e.g., those cited above, to identify additional examples of the compounds of the present invention.

Compounds of the present invention can further be used to design more effective
30   analogs using modeling packages such as Ludi, Insight II, C^2-Minimizer and Affinity (Molecular Simulations Inc., San Diego, CA). A particularly preferred modeling package is MacroModel (Columbia University, NY,NY).

The compounds of the present invention can further be used as the basis for developing a rational combinatorial library. Such a library can also be screened to identify
35   more effective compounds. While the nature of the combinatorial library is dependent on various factors such as the particular compound chosen from the preferred compounds of the present invention to form the basis of the library, as well as the desire to synthesize the library

-33-

using a resin, it will be recognized that the compounds of the present invention provide requisite data suitable for combinatorial design programs such as C^2-QSAR (Molecular Simulations Inc., San Diego, CA).

5   Another class of compounds that can be used to inhibit the function of P protein are P protein-encoding gene antisense nucleic acids. A P protein-encoding gene antisense nucleic acid as used herein refers to an oligonucleotide or polynucleotide molecule having a nucleic acid sequence capable of hybridizing to a portion of a P protein-encoding RNA (preferably mRNA) by virtue of some degree of sequence complementarity. The antisense nucleic acid should be complementary to either a coding and/or noncoding region of a P protein mRNA

10   such that it inhibits P protein function by reducing the amount of P protein synthesized.

      The antisense nucleic acids of the present invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA, or a modification or analog thereof, which can be directly administered to a cell, or to the skin of an animal, or which can be produced intracellularly by transcription of heterologous, introduced sequences.

15   In one embodiment, the present invention is directed to methods for inhibiting the expression of a P protein-encoding nucleic acid sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising a P protein-encoding gene antisense nucleic acid of the present invention.

      The P protein-encoding gene antisense nucleic acids of the present invention are at

20   least about six nucleotides in length and are more preferably oligonucleotides ranging from about 6 to about 50 oligonucleotides. In specific aspects, the oligonucleotide is at least about 10 nucleotides, at least about 15 nucleotides, at least about 100 nucleotides, or at least about 200 nucleotides in length. The oligonucleotides can be DNA or RNA, or chimeric mixtures or derivatives, and modified versions thereof, which can either be single-stranded or

25   double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone level. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988),

30   hybridization-triggered cleavage agents (see, e.g., Krol et al., 1988, BioTechniques 6:958-976), or intercalating agents (see, e.g., Zon, 1988, Pharm. Res. 5:539-549).

      In a preferred aspect of the present invention, a P protein-encoding gene antisense oligonucleotide is a single-stranded DNA molecule. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

35   The P protein-encoding gene antisense oligonucleotide may comprise at least one modified base moiety which is selected from a group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine,

-34-

5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine,

5   7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5N-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil,

10   3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from a group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified

15   phosphate backbone component selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an alpha-anomeric oligonucleotide. An alpha-anomeric oligonucleotide forms specific double-stranded hybrids with

20   complementary RNA in which, contrary to the usual beta-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule such as, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

25   Oligonucleotides of the present invention may be synthesized by standard methods known in the art including, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al., 1988, Nucl. Acids Res. 16:3209, and methylphosphonate oligonucleotides can be prepared by use

30   of controlled pore glass polymer supports using the method of Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451, etc.

In a specific embodiment, the P protein antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). In another embodiment, the

35   oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

-35-

In an alternative embodiment, the P protein-encoding gene antisense nucleic acid of the invention is produced intracellularly by transcription from an heterologous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the

5   invention. Such a vector would contain a sequence encoding the P protein-encoding gene antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by standard recombinant DNA technology methods known in the art. Vectors can be plasmids, viral vectors, or others known in the art as useful for replication

10 and expression in mammalian cells. Expression of the sequence encoding the P protein-encoding gene antisense RNA can be regulated by any promoter known in the art to act in such cells. Such promoters can be inducible or constitutive, and can include but are not limited to those listed above.

The antisense nucleic acids of the invention comprise a sequence complementary to

15 at least a portion of an RNA transcript of a P protein-encoding gene, preferably a human P protein-encoding gene. However, absolute complementarity, although preferred, is not required, as long as the antisense nucleic acid has sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex. In the case of double-stranded P protein-encoding gene antisense nucleic acids, a single strand of the duplex DNA may thus

20 be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a P protein-encoding gene RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can determine the mismatch tolerance by use of standard procedures to, *e.g.*,

25 determine the melting point of the hybridized complex.

5.3   Methods of Inhibiting, Increasing or Mimicking P Protein Function

Compounds that affect or mimic the function of P protein can be used to treat animals or, preferably, humans that have diseases, conditions, or disorders caused by the production or overproduction of melanin. Such diseases, conditions, or disorders include those that can

30 be characterized by discolorations of the skin or hair such as, for example, hyperpigmentation caused by inflammation or from diseases such as melasma, or brown spots such as "café au lait" macules. Alternatively, a subject may wish to lighten the color of his or her hair or skin. Compounds that increase the function of P protein or that mimic the function of P protein can be used to treat animals or, preferably, humans that have diseases, conditions, or disorders

35 caused by the underproduction of melanin such as, for example, post-inflammatory hypopigmentation, pityriasis alba, and certain forms of albinism such as, for example, OCA II albinism. Additionally, such compounds can be used to darken the color of one's hair or skin.

-36-

For the purposes of this application, the terms "treatment", "therapeutic use", and "medicinal use" shall refer to any and all uses of the compositions of the invention which remedy a disease state or one or more symptoms, or otherwise prevent, hinder, retard, or reverse the progression of disease or one or more other undesirable symptoms in any way
5   whatsoever.

5.3.1   Pharmaceutical Applications

For pharmaceutical uses, it is preferred that the compound that affects or mimics P protein function is part of a pharmaceutical composition. Pharmaceutical compositions, comprising an effective amount of a compound that affects P protein function in a
10   pharmaceutically acceptable carrier, can be administered to a patient, person, or animal having a disease, disorder, or condition which is of a type that produces, or overproduces, melanin.

The amount of compound that affects or mimics P protein function which will be effective in the treatment of a particular disease, disorder, or condition will depend on the
15   nature of the disease, disorder, or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine *in vitro* the cytotoxicity of the compound to the tissue type to be treated, and then in a useful animal model system prior to testing and use in humans.

The compounds that affect or mimic P protein function can be administered for the
20   reduction or increase of melanin synthesis by any means that results in contact of the active agent with its site of action in the body of a mammal. The compounds can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic agents or in a combination of therapeutic agents. Each can be administered alone, but is preferably administered with a pharmaceutical carrier selected on
25   the basis of the chosen route of administration and standard pharmaceutical practice. The pharmaceutical compositions of the invention can be adapted for oral, parenteral, topical or rectal administration, and can be in unit dosage form, in a manner well known to those skilled in the pharmaceutical art. Parenteral administration includes but is not limited to, injection subcutaneously, intravenously, intraperitoneally or intramuscularly. However, topical
30   application is preferred.

5.3.2   Cosmetic Applications

In addition to pharmaceutical uses, the methods of the current invention are useful for cosmetic purposes. Cosmetic applications for methods of the present invention include the topical application of compositions containing one or more compounds that affect or mimic P
35   protein function to enhance or otherwise alter the visual appearance of skin or hair. Occurrences in the skin or hair of noticeable but undesired pigmentation as a result of

-37-

melanin production, overproduction or underproduction can be treated using the methods of the present invention.

5.3.3   Endpoints and Dosages

An effective dosage and treatment protocol can be determined by conventional
5   means, starting with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well.   Animal studies, preferably mammalian studies, are commonly used to determine the maximal tolerable dose, or MTD, of a bioactive agent per kilogram weight.   Those skilled in the art can extrapolate doses for efficacy and avoidance of toxicity to other species, including humans.

10   Before human studies of efficacy are undertaken, Phase I clinical studies in normal subjects can help establish safe doses.   Numerous factors can be taken into consideration by a clinician when determining an optimal dosage for a given subject.   Primary among these is the toxicity and half-life of the chosen compound that affects or mimics P protein function. Additional factors include the size of the patient, the age of the patient, the general condition
15   of the patient, the particular disease, condition, or disorder being treated, the severity of the disease, condition, or disorder being treated, the presence of other drugs in the patient, the effect desired, and the like.   The trial dosages would be chosen after consideration of the results of animal studies and the clinical literature.

One of ordinary skill in the art will appreciate that the endpoint chosen in a particular
20   case will vary according to the disease, condition, or disorder being treated, the outcome desired by the patient, subject, or treating physician, and other factors.   Where the composition is being used to lighten or darken skin color such as, for example, to reverse hyperpigmentation caused by, for example, inflammation or diseases such as melasma, or to lighten or darken hair color, any one of a number of endpoints can be chosen.   For example,
25   endpoints can be defined subjectively such as, for example, when the subject is simply "satisfied" with the results of the treatment.   For pharmacological compositions, the endpoint can be determined by the patient's, or the treating physician's, satisfaction with the results of the treatment.   Alternatively, endpoints can be defined objectively.   For example, the patient's or subject's skin or hair in the treated area can be compared to a color chart.   Treatment is
30   terminated when the color of the skin or hair in the treated area is similar in appearance to a color on the chart.   Alternatively, the reflectance of the treated skin or hair can be measured, and treatment can be terminated when the treated skin or hair attains a specified reflectance. Alternatively, the melanin content of the treated hair or skin can be measured.   Treatment can be terminated when the melanin content of the treated hair or skin reaches a specified value.
35   Melanin content can be determined in any way known to the art, including by histological methods, with or without enhancement by stains for melanin.

-38-

5.3.4   Methods of Administration

The compound that affects or mimics P protein function (*i.e.*, the active ingredient) can be administered orally in solid or semi-solid dosage forms, such as hard or soft-gelatin capsules, tablets, or powders, or in liquid dosage forms, such as elixirs, syrups, or
5   suspensions. It can also be administered parenterally, in sterile liquid dosage forms. Since topical application is preferred, other dosage forms are potentially possible such as patches, ointments, creams, gels, lotions, solutions, suppositories or transdermal administration.

Because *in vivo* use is contemplated, the composition is preferably of high purity and substantially free of potentially harmful contaminants, *e.g.*, at least National Food (NF) grade,
10   generally at least analytical grade, and preferably at least pharmaceutical grade. To the extent that a given compound must be synthesized prior to use, such synthesis or subsequent purification shall preferably result in a product that is substantially free of any potentially contaminating toxic agents that may have been used during the synthesis or purification procedures.

15   Gelatin capsules or liquid-filled soft gelatin capsules can contain the active ingredient and powdered or liquid carriers, such as lactose, lecithin starch, cellulose derivatives, magnesium stearate, stearic acid, and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed
20   tablets can be sugar-coated or film-coated to mask any unpleasant taste and to protect the tablet from the atmosphere, or enteric-coated for selective, targeted disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and/or flavoring to increase patient acceptance.

In general, sterile water, oil, saline, aqueous dextrose (glucose), polysorbate and
25   related sugar solutions and glycols such as propylene glycol or polyethylene glycols, are suitable carriers for parenteral solutions. Solutions or emulsions for parenteral administration preferably contain about 5-15% polysorbate 80 or lecithin, suitable stabilizing agents, and if necessary, buffer substances. Antioxidizing agents, such as but not limited to sodium bisulfite, sodium sulfite, or ascorbic acid, either alone or combined, are suitable stabilizing
30   agents. Also useful are citric acid and its salts, and sodium EDTA. In addition, parenteral solutions can contain preservatives, including but not limited to benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol.

Suitable pharmaceutical carriers are further described in *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Company, Easton, PA (1990) a standard reference text
35   in this field, which is incorporated herein by reference in its entirety.

Useful pharmaceutical dosage forms for administration of compounds that affect or mimic P protein function are described below.

For topical administration, compounds that affect or mimic P protein function can be formulated as a solution, gel, lotion, ointment, cream, suspension, paste, liniment, powder, tincture, aerosol, transdermal drug delivery system, or the like in a pharmaceutically or cosmetically acceptable form by methods well known in the art. The composition can be any
5   of a variety of forms common in the pharmaceutical or cosmetic arts for topical application to animals or humans, including solutions, lotions, sprays, creams, ointments, salves, gels, etc. Preferred agents are those that are viscous enough to remain on the treated area, those that do not readily evaporate, and/or those that are easily removed by rinsing with water, optionally with the aid of soaps, cleansers and/or shampoos. Actual methods for preparing
10 topical formulations are known or apparent to those skilled in the art, and are described in detail in *Remington's Pharmaceutical Sciences*, 1990 (above); and *Pharmaceutical Dosage Forms and Drug Delivery Systems*, 6th ed., Williams & Wilkins (1995).

In order to enhance the percutaneous absorption of the active ingredients, one or more of a number of agents can be added in the topical formulations, including but not limited
15 to dimethylsulfoxide, dimethylacetamide, dimethylformamide, surfactants, azone, alcohol, acetone, propylene glycol and polyethylene glycol. In addition, physical methods can also be used to enhance transdermal penetration such as, *e.g.*, by iontophoresis or sonophoresis. Alternatively, or in addition, liposomes may be employed.

The pharmaceutical compositions can be applied directly to the skin. Alternatively,
20 they can be delivered by various transdermal drug delivery systems, such as transdermal patches as known in the art.

The invention having been described, the following examples are offered by way of illustration and not limitation.

6.   Example: Targeting Function Screen
25   In this example, the effect of P protein on cellular targeting of tyrosinase was investigated. This function was then exploited in a screen for compounds that inhibit the activity of P protein.

6.1   Materials and Methods
Melan-a cells (*a/a, P/P*), an immortalized melanocyte line derived from C57BLI6J
30 mice wildtype at the *p* locus (Bennett *et al.*, 1987, Int. J. Cancer 39:414-418), were maintained in culture in Dulbecco's modification of Eagle's medium (DME). Melan-p1 melanocytes from mice lacking all *p* gene transcripts due to the presence of overlapping deletions (*a/a,* p^{cp}/p^{25H}) (Sviderskaya *et al.*, 1997, J. Invest. Dermatol. 108:30-34) were maintained in Ham's F10 medium. Both media were supplemented with 10% fetal calf serum, 5% sodium pyruvate, 5%
35 glutamate, 5 units/ml penicillin, 5 μg/ml streptomycin, 1% non-essential amino acids and 200 nM 12-0-tetradecanoyl phorbol 13-acetate. In addition, 200 pM cholera toxin was added to the melan-p1 cells.

-40-

Cells were maintained in the appropriate media, which was then replaced with tyrosine deficient DME medium (DME-D) supplemented with either 0.03 mM tyrosine for low tyrosine conditions or 0.3 mM tyrosine for high tyrosine conditions (Bennett, D.C. et al., 1987, Int. J. Cancer 39:414-418), (Sviderskaya et al., , J. Invest. Dermatol. 108:30-34). Aliquots of
5   culture medium were withdrawn, dialyzed against 0.1 M sodium phosphate buffer, pH 6.8, and analyzed for tyrosinase activity using a radiometric tyrosine hydroxylase assay (Orlow, S.J. et al., 1990, J. Invest. Dermatol. 94:461-64).

For treatment with test compounds, cultured melan-a melanocytes were incubated for 48 hours in the presence of low tyrosine in the medium as above but in the presence of
10   benztropine (10 micromolar final concentration), or imipramine (10 micromolar final concentration), or nitroquipazine, (30 micromolar final concentration), or left untreated. Incubation media were assayed for tyrosinase activity, as above.

6.2   Results

Increasing tyrosinase activity in the media removed from melan-p cell cultures grown
15   in the presence of low tyrosine indicates that these cells secrete relatively large amounts of tyrosinase into their incubation media (FIG. 1). By contrast, melan-a cells, which represent wildtype melanocytes, secrete significantly less tyrosinase into the media (FIG. 1). While culture in the presence of excess tyrosine had little effect on melan-a cells, the amount of enzyme secreted by melan-p1 cells was reduced. As predicted above, tyrosine appears to
20   partially correct the misrouting of tyrosinase in melan-p1 cells.

Treatment with benztropine did not alter the levels of tyrosinase activity secreted to the incubation medium of melan-a cells (FIG. 2). Treatment with either imipramine or nitroquipazine significantly increased the levels of tyrosinase activity found in the cells' incubation medium (FIG. 2).

25   6.3   Discussion

Melan-a cells are melanocytes derived from wildtype mice. They have fully functional P protein and tyrosinase, and produce melanin. Melan-p cells, however, are derived from p-null mice having a deletion of the entire p gene coding sequence. Thus, they produce no P protein. Consequently, melan-p cells have lower tyrosinase activity and make less melanin
30   than melan-a cells.

This example, which can be performed with any type of melanogenic cell, demonstrates that melanocytes lacking P protein function secrete significantly more tyrosinase into their growth or incubation medium than do melanocytes with normal P protein function. This result is obtained either when the cells are genetically altered to reduce or
35   eliminate P protein function, as in melan-p cells (FIG. 1), or when the cells are treated with a compound that inhibits P protein function, such as imipramine (FIG. 2b).

-41-

7.   Example: Tyrosinase Activity Screen

In this example, the effect of P protein on the measurable enzymatic activity of tyrosinase from cells genetically engineered to express tyrosinase was investigated. Any melanogenic cell type that expresses both P protein and tyrosinase, or any cell type made to

5   express both P protein and tyrosinase, can be substituted. This function was then exploited in a screen for compounds that inhibit the function of P protein.

7.1   Materials and Methods

Cultured melan-a melanocytes, as described above in Section 6, were incubated for 48 hours in the presence of benztropine (10 micromolar final concentration), or imipramine

10   (10 micromolar final concentration), or nitroquipazine (30 micromolar final concentration), or left untreated. Cells were washed and extracted with 50mM Tris-HCl (pH 7.4), 2mM EDTA, 150 mM NaCl and 1% Triton X-100. Cell extracts were analyzed for tyrosinase activity using a radiometric tyrosine hydroxylase assay (Orlow, S.J. et al., 1990, above).

Expression vectors were constructed to express P protein and tyrosinase genes in

15   cultured cells. Specifically, the coding sequence for tyrosinase was removed as a HindIII-EcoRI fragment from clone TYBS (Yokohama et al., 1990, Nucl. Acids. Res. 18:7293-7298) and cloned into the HindIII/EcoRI sites of pcDNA I/amp (Invitrogen, CA). Coding sequence for the P protein was removed as a BamHI-EcoRV fragment from MC2701 (Gardner et al., 1992, above) and cloned into the BamHI/EcoRV sites of pcDNA3 and pcDNA3.1/V5/His-

20   TOPO (Invitrogen, CA). COS cells were transfected with the pcDNA1-based plasmids and FuGENE™ 6 (Roche Molecular Biochemicals, Indianapolis, IN) as transfection agents for 48 hours. Cells were transformed with: (i) the vector alone; (ii) the vector carrying a tyrosinase-encoding gene; (iii) the vector carrying a P protein-encoding gene; or (iv) vectors carrying a tyrosinase-encoding gene and a P protein-encoding gene. Transformed cells were washed

25   and extracted as above. Tyrosinase activity was then measured as above. Tyrosinase assays were performed on 60 micrograms of cell protein.

COS cells transfected with a vector carrying a tyrosinase-encoding gene, or with vectors carrying a tyrosinase-encoding gene and a P protein-encoding gene as above, were treated with benztropine, or imipramine, or nitroquipazine, or left untreated, as above, and cell

30   extracts were then prepared as above. The tyrosinase activity of cell extracts was determined as above.

7.2   Results

As shown in FIG. 2a, extracts from melan-a cells treated with benztropine or nitroquipazine had greater tyrosinase activities than untreated cells. Extracts from cells

35   treated with imipramine had less tyrosinase activity than untreated cells.

As shown in FIG. 3, extracts from COS cells transfected with the vector alone (V+V) or with the vector carrying the P protein-encoding gene (V+P) did not exhibit measurable

-42-

tyrosinase activity. Extracts from cells transfected with the vector carrying the tyrosinase-encoding gene (V+T) had measurable tyrosinase activity, while extracts from cells transfected with the vectors carrying the tyrosinase-encoding gene and the P protein-encoding gene (T+P) had tyrosinase activity approximately four fold greater than the

5   tyrosinase activity found in extracts of cells transfected with the vector carrying the tyrosinase-encoding gene alone (V+T).

FIG. 4 shows the separate effects of three compounds on P protein function. Nitroquipazine (4) caused extracts from tyrosinase-expressing COS cells to exhibit lower tyrosinase activity, regardless of whether the cells were expressing the P protein.

10   Benztropine (2) did not have an appreciable effect on tyrosinase activity in these extracts. Imipramine (3) dramatically reduced the tyrosinase activity of cells expressing both P protein and tyrosinase, but had very little effect on cells expressing only tyrosinase.

7.3   Discussion

This example illuminates the relationship between P protein function and tyrosinase

15   activity in cell extracts. Melanocytes that express P protein can be made to mimic cells that lack P protein function through the use of compounds that inhibit P protein function. Melan-a cells are wildtype for the P protein-encoding gene. Yet extracts taken from these cells after they are treated with imipramine have lower tyrosinase activity than untreated melan-a cells (FIG. 2). In contrast, extracts from cells treated with benztropine or nitroquipazine have

20   higher tyrosinase activity than untreated cells (FIG. 2).

COS cells are derived from monkey kidney cells. Normally, they do not express tyrosinase or P protein. This example demonstrates that by transfecting COS cells with a tyrosinase-encoding gene and a P protein encoding gene, one can produce what might be considered an "artificial melanocyte." These cells express active tyrosinase and P protein

25   (FIG. 3), and even produce melanin. Cotransfection of COS cells with both a tyrosinase-encoding gene and a P protein-encoding gene produces cells with approximately four times more tyrosinase activity than COS cells transfected with a tyrosinase-encoding gene alone (FIG. 3). This result demonstrates that P protein is expressed and active in these cells because the intracellular activity of tyrosinase was increased by P protein expression.

30   Extracts from COS cells that have been transformed with both a tyrosinase-encoding gene and a P protein-encoding gene and then treated with imipramine contained only about one third of the tyrosinase activity of similar cells not treated with imipramine (FIG. 4). The tyrosinase activity of COS cells that were transfected with only a tyrosinase-encoding gene and then treated with imipramine was not significantly different than the tyrosinase activity of

35   extracts of similar cells not treated with imipramine (FIG. 4). These results indicate that imipramine reduces tyrosinase activity by inhibiting P protein function. By contrast, benztropine did not reduce the tyrosinase activity of extracts of transfected COS cells,

whether or not they expressed P protein (FIG. 4). In addition, nitroquipazine reduced the tyrosinase activity of extracts of transfected COS cells, whether or not they expressed P protein (FIG. 4). This result indicates that nitroquipazine is not an inhibitor of P protein function.

8.   Example: Secretion of Tyrosinase in Melan-p Cells Results from Proteolysis

While we observed activity of tyrosinase in the medium, Potterf et al. (1998, Exp. Cell Res. 244:319-326) did not detect tyrosinase protein in the medium using αPEP7. Tyrosinase is a type I membrane protein anchored in the membrane, and it is thus likely that proteolysis, which leads to the clipping of the tail, is required for secretion. The truncated protein would not be detected by αPEP7, which is directed against the tail, although the catalytic domains would remain functional. We therefore, examined the effects of a series of protease inhibitors on the secretion of tyrosinase by melan-a and melan-p1 cells. E64, an epoxysuccinyl peptide and a potent inhibitor of cysteine proteinases was found to be the most effective in reducing the amount of tyrosinase secreted into the media of melan-p1 cells (FIG. 5a), thus demonstrating that secretion of tyrosinase can be inhibited by blocking the activity of cysteinyl proteases.

If proteolysis and secretion of tyrosinase were the precipitating factor in the misrouting of tyrosinase, then E64 should increase melanin accumulation in melan-p1 cells. The effects of E64 were further investigated, and a potential synergy with tyrosine, which also reduced secretion into the media, examined. A range of E64 concentrations was tested at low (0.03 mM) and high (0.3 mM) tyrosine.

At 0.03 mM tyrosine, 12.5 μM E64 lowered secretion of tyrosinase into the medium from 7.1% to 4.0% (FIG. 5a), whereas at higher concentrations (25 μM), E64 was only slightly more effective (3.8% activity in media). E64 also reduced tyrosinase secretion at higher tyrosine concentrations (0.3 mM), reducing the tyrosinase in the medium from 6.5% to 3.9%. The higher concentration of E64 was not more effective. Surprisingly, E64 reduced intracellular melanin production at high concentrations of tyrosine. Thus, despite its ability to diminish proteolysis and secretion of tyrosinase from melan-p1 cells, E64 was not able to cause tyrosinase to re-route to the melanosome and begin melanin synthesis and deposition.

9.   Example: Comparison of Ultrastructure and Distribution
        of Tyrosinase in Melan-a and Melan-p1 Cells

        9.1   Materials and Methods

Melanocytes were seeded into Lab-Tek chamber slides (Nunc, Inc., Naperville, Il) and grown to 90% confluence. Cultured melanocytes were fixed in wells with half-strength Karnovsky's fixative (Karnovsky, 1965) in 0.2 M sodium cacodylate buffer at pH 7.2 for 30 minutes at room temperature. For dihydroxyphenylalanine (DOPA) histochemistry, fixed cells were incubated in 0.1% 1-DOPA twice for 2.5 hours. The cells were washed 3 times in buffer

-44-

and treated with 1.0% osmium tetroxide containing 1.5% potassium ferrocyanide (Karnovsky, 1971) for 30 minutes. The cells were washed, stained en bloc with 0.5% uranyl acetate for 30 minutes, dehydrated, and embedded in Eponate 12. Areas of the Epon case were cut out and mounted on Epon pegs and sectioned on an RMC MT 6000-XL ultramicrotome. Ultrathin

5 sections were stained with aqueous solutions of uranyl acetate (2%) and lead citrate (0.3%) for 15 minutes each, and then viewed and photographed in a JEOL JEM-IOOCX transmission electron microscope.

9.2   Results

Previous studies have shown that deficiency of the P protein results in both

10 ultrastructural aberrations (Moyer, 1966, Am Zool 6:43-66; Sidman and Pearlstein, 1965, Dev. Biol. 12:93-116; Orlow and Brilliant, 1999, Exp. Eye Res. 68:147-154) as well as abnormal subcellular localization of tyrosinase (Potterf et al., 1998, supra). In order to investigate both features simultaneously, we determined the subcellular architecture and the distribution of tyrosinase in melan-a and melan-p1 cells by electron microscopy with and without DOPA

15 histochemistry.

As reported previously (Rosemblat et al., 1998, Exp. Cell Res. 239:344-352), cultured melan-a cells, wildtype at the p locus, contained melanosomes that were predominantly of stage IV maturation (FIG. 6a). In contrast, p-null melan-p1 cells exhibited melanosomes that were predominantly stage I and II and occasionally stage III (FIG. 6b).

20 Upon DOPA incubation of melan-a cells, tyrosinase activity was demonstrated in the trans Golgi network (TGN) and in 50 nm vesicles which were confined to the vicinity of the Golgi apparatus (FIG. 7a). DOPA treated melan-p1 cells also demonstrated reaction product in the TGN and neighboring 50 nm vesicles (FIG. 7b). In addition, reaction product was present in some melan-p1 melanosomes. However, many melanosomes, both in the cell body

25 as well as in the dendrites, remained devoid of reaction product (FIG. 7b). Unlike melan-a cells (FIG. 7a), melan-p1 cells exhibited reaction product in 50 nm vesicles well outside of the peri-nuclear Golgi area (FIG. 7b) and in close proximity to the plasma membrane (FIG. 7b) suggesting an abnormal accumulation of tyrosinase in a population of vesicles.

9.3   Discussion

30 The lack of P protein resulted in the proliferation of small tyrosinase-containing vesicles that were no longer limited to the area around the TGN. Tyrosinase was therefore either packaged into different vesicles in the two cell lines or, alternatively, the vesicles were the same, but their routing was disrupted in the absence of P. Tyrosinase in these aberrant vesicles could be detected by DOPA staining and was thus enzymatically active. The increase

35 in mature melanosomes in melan-p1 cells cultured in high tyrosine was not accompanied by a major reduction in the number of 50 nm vesicles, suggesting partial, but not complete, correction, of the p phenotype by tyrosine.

-45-

10.   Example: Targeting of Lysosomal Hydrolases in Melan-a and Melan-p Cells

This experiment demonstrates that melan-p cells do not properly target a certain class
of lysosomal hydrolases to the lysosome.

10.1   Material and Methods

Melan-a and melan-p cells as described above in Section 6 were seeded to high
density and grown in low tyrosine (14 μM) DME medium. Large granule and small granule
fractions were prepared and centrifuged on pre-layered sucrose gradients as described in
Rosemblatt *et al.*, 1994, above and Seiji, 1963, Annals N.Y. Acad. Sci., 100:497-533.
Fractions were collected from the top down.

Appropriate reaction substrates for the lysosomal enzyme assays prepared in 0.2 M
sodium acetate, 1% TritonX-100 were as follows:

β-hexosaminidase	- 4 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide
β-glucosidase	- 4.6 mM 4-methylumbelliferyl-N-acetyl-β-D-glucoside
β-glucuronidase	- 4.6 mM 4-methylumbelliferyl-N-acetyl-β-D-glucoronide
β-galactosidase	- 4.6 mM 4-methylumbelliferyl-N-acetyl-β-D-galactoside
Acid phosphatase	- 22.5 mM 4-methylumbelliferyl-phosphate

The reaction mix was prepared in 96 well flat bottom plates. Each well was loaded
with 25 μl of a gradient fraction, 2.5 μl 1M sodium acetate and 27.5 μl of the appropriate
substrate mix.   The plates were covered with parafilm and incubated at 37°C.   β-
hexosaminidase reactions were incubated for 50 minutes, β-glucosidase, β-glucuronidase and
β-galactosidase reactions were incubated for 20 minutes, and acid phosphatase reactions
were incubated for 10 minutes. Reaction was stopped by addition of 200 μl of stop buffer
(132 mM glycine, 68 mM sodium chloride, 83 mM anhydrous sodium carbonate), and plates
read immediately using an excitation wavelength of 370 nm and an emission wavelength of
460 nm.

10.2   Results

In both melan-a and melan-p cells, very little of the lysosomal hydrolases were
detected in the small granule fraction (see FIGS. 8-12). This result was expected because
the small granule fraction consisted of mostly small vesicles in which lysosomal hydrolases do
not normally accumulate. The large granule fraction contains endoplasmic reticulum, Golgi
organelles, lysosomes and melanosomes and, hence, should contain most of the lysosomal
hydrolases. With respect to acid phosphatase, there was only slightly less overall activity for
the enzyme in large granule fractions from melan-p cells as compared to those from melan-a
cells (FIG. 8B). Additionally, there was a minor shift in localization of acid phosphatase to
slightly less dense fractions in the melan-p cells as compared to melan-a cells. However, with
respect to the other lysosomal hydrolases assayed, the differences between melan-a and
melan-p cells was dramatic. In fact, the overall activity of β-hexosaminidase, β-glucosidase,

-46-

β-glucuronidase and β-galactosidase was significantly reduced in melan-p cells as opposed to melan-a cells (see FIGS. 9-12, right panels). This loss of activity could not be attributed to a shift of the enzymes within the cells because whole cell extracts demonstrated the similar significant decreases in activity of β-hexosaminidase, β-glucosidase, β-glucuronidase and β-
5   galactosidase in melan-p cells as opposed to melan-a cells, but with essentially no difference in the total amounts of alkaline phosphatase between melan-p and melan-a cells (results not shown). While the same large granule fractions from melan-a cells that contained acid phosphatase also contained most of the β-hexosaminidase, β-glucosidase, β-glucuronidase and β-galactosidase activities, melan-p cells had a significant reduction in activity of these
10   enzymes in the large granule fractions. Thus, β-hexosaminidase, β-glucosidase, β-glucuronidase and β-galactosidase enzymes do not accumulate correctly in lysosomes in melan-p cells.

　　　　　10.3   Discussion

　　　　　Unlike acid phosphatase, the enzymes β-hexosaminidase, β-glucosidase, β-
15   glucuronidase and β-galactosidase are not transported to the cell surface prior to eventually reaching the lysosome. Instead, these enzymes are transported from the *trans*-Golgi network to the late endosome via the activity of the M6P/IGF-II receptor. The differences in targeting of these two classes of lysosomal hydrolases in melan-p cells versus melan-a cells indicates that disruption of P protein function affects M6P/IGF-II receptor-mediated targeting. Based on
20   our results showing the secretion of tyrosinase from melan-p cells, and the intracellular depletion of β-hexosaminidase, β-glucosidase, β-glucuronidase and β-galactosidase in large granule fractions from the same cells, this class of lysosomal enzymes should be secreted from the melan-p cells. Accordingly, targeting of these enzymes, assayed by an increase in secretion or a reduction in accumulation in lysosomal membrane fractions, can also be used
25   as part of an assay to screen for compounds that affect the function of P protein.

EQUIVALENTS

　　　　　The foregoing written specification is sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described means for carrying out the invention which are obvious to those skilled in the field of molecular biology,
30   medicine or related fields are intended to be within the scope of the following claims.

　　　　　All patents, patent applications, and publications cited above are incorporated herein by reference in their entirety.

-47-

CLAIMS

What is claimed is:

1.   A method of screening for compounds that inhibit melanogenesis, the method comprising: treating cells expressing a tyrosinase-encoding gene with a test compound, and determining the cellular localization of tyrosinase in the presence of the test compound; wherein a change in the cellular localization of tyrosinase in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

2.   The method of claim 1, wherein the cells further express a P protein-encoding gene, and wherein the change in the cellular localization of tyrosinase in the presence of the test compound as compared to in the absence of the test compound is dependent upon the expression of the P protein-encoding gene.

3.   The method of claim 1 or 2, wherein the cellular localization of tyrosinase is determined by assaying the amount of tyrosinase secreted by the cells in the presence of the compound, wherein an increase in the amount of tyrosinase secreted by the cells in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

4.   The method of claim 1 or 2, wherein the cellular localization of tyrosinase is detected by assaying for tyrosinase activity.

5.   The method of claim 1 or 2, wherein the cellular localization of tyrosinase is detected by assaying for the presence of tyrosinase protein using immunological techniques.

6.   The method of claim 1 or 2 further comprising the step of assaying the amount of tyrosinase associated in a high molecular weight complex in the presence of the test compound, wherein a decrease in the amount of tyrosinase associated in a high molecular weight complex in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

7.   The method of claim 1 or 2 further comprising the step of assaying the amount of TRP-1 or TRP-2 protein associated in a high molecular weight complex in the presence of the compound, wherein a decrease in the amount of TRP-1 or TRP-2 protein associated in a high molecular weight complex in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

8.   The method of claim 1 or 2 further comprising the step of assaying the number or size of melanosomes in the cells in the presence of the compound, wherein a decrease in the number or size of melanosomes in the cells in the presence of the test

-48-

compound as compared to in the absence of the test compound indicates that the test compound inhibits melanogenesis.

9.   The method of claim 1 or 2 further comprising the step of assaying the mass or length of tyrosinase in the cells in the presence of the compound, wherein a decrease in the mass or length of tyrosinase in the cells in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

10.   The method of claim 1 or 2 further comprising the step of assaying for the levels and/or targeting of lysosomal hydrolases in the cells in the presence of the compound, wherein a decrease in accumulation of lysosomal hydrolases that are transported via the M6P/IGF-II receptor in the lysosome in the cells in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that inhibits melanogenesis.

11.   The method of claim 1 or 2, wherein the cells are grown in the presence of low tyrosine.

12.   The method of claim 11 wherein the concentration of tyrosine is 0.01-0.03 mM.

13.   The method of claim 1 wherein the cells are melanocytes.

14.   The method of claim 1 wherein the cells are melanoma cells.

15.   The method of claim 1 wherein the cells are derived from a mammal.

16.   The method of claim 15 wherein the mammal is a human.

17.   The method of claim 15 wherein the mammal is selected from the group consisting of mouse, hamster, and guinea pig.

18.   A method of screening for compounds that increase melanogenesis comprising: treating cells expressing a tyrosinase-encoding gene with a test compound, and determining the amount of tyrosinase secreted by the cells in the presence of the test compound; wherein a decrease in the amount of tyrosinase secreted by the cells in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that increases melanogenesis.

19.   The method of claim 18 wherein the cells further express a P protein-encoding gene, and wherein the decrease in the amount of tyrosinase secreted by the cells in the presence of the test compound as compared to in the absence of the test compound is dependent upon the expression of the P protein-encoding gene.

20.   The method of claim 18 or 19 further comprising determining a ratio of tyrosinase inside the cells to tyrosinase secreted by the cells, wherein an increase in the ratio in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound induces melanogenesis.

-49-

21.   The method of claim 18 or 19, wherein the amount of tyrosinase is detected by assaying for tyrosinase activity.

22.   The method of claim 18 or 19, wherein the amount of tyrosinase is detected by assaying for the presence of tyrosinase protein using immunological techniques.

23.   The method of claim 18 wherein the cells are melanocytes.

24.   The method of claim 18 wherein the cells are melanoma cells.

25.   The method of claim 23 or 24, wherein the cells are visually examined for an increase in melanin production.

26.   The method of claim 23 or 24 wherein the cells do not express P protein, and wherein a decrease in the amount of tyrosinase secreted by the cells in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is a candidate for a compound that mimics P protein function.

27.   The method of claim 23 wherein the cells are mouse melan-p melanocytes.

28.   The method of claim 18 wherein the cells are derived from a mammal.

29.   The method of claim 28 wherein the mammal is a human.

30.   The method of claim 28 wherein the mammal is selected from the group consisting of mouse, hamster, and guinea pig.

31.   The method of claim 26, wherein the cells are visually examined for an increase in melanin production.

32.   A method of screening for compounds that affect the function of P protein, the method comprising: contacting a system with a test compound, the system comprising P protein and tyrosinase; and identifying those test compounds that affect tyrosinase activity in the system in a P protein-dependent manner.

33.   The method of claim 32 wherein the system is a cell that expresses a P protein-encoding gene and a tyrosinase-encoding gene.

34.   The method of claim 33 wherein the cell is a cultured cell.

35.   The method of claim 32 wherein compounds that decrease tyrosinase activity in the system are identified as compounds that inhibit the function of P protein.

36.   The method of claim 35 further comprising the step of assaying for the targeting of lysosomal hydrolases in the cells in the presence of the compound, wherein a decrease in accumulation of lysosomal hydrolases that are transported via the M6P/IGF-II receptor in the lysosome in the cells in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound inhibits the function of P protein.

37.   The method of claim 32 wherein compounds that result in an increase in tyrosinase activity in the system are identified as compounds that increase the function of P protein.

-50-

38.   The method of claim 32 wherein the P protein-encoding gene is derived from a mammal selected from the group consisting of human, hamster, guinea pig, and mouse.

39.   The method of claim 32 wherein the tyrosinase-encoding gene is derived from a mammal selected from the group consisting of human, hamster, guinea pig, and
5   mouse.

40.   A method of screening for compounds that affect the function of P protein, the method comprising: using a primary screening method to preliminarily determine whether a test compound may affect P protein function; and using one or more secondary screening methods to determine whether the test compound affects P protein function.

10   41.   The method of claim 40, wherein the primary screening method comprises at least one screening assay selected from the group consisting of assaying for secretion of tyrosinase and assaying for the missorting of at least one lysosomal hydrolase.

42.   A method of screening for compounds that affect the function of P protein, the method comprising: modeling a compound that affects the function of the P protein; making
15   chemical analogs of the compound; and assaying the chemical analogs for their effect on the function of P protein.

43.   The method of claim 42 wherein the compound is imipramine.

FIG. 1

FIG. 2B

Tyrosinase activity in media from Mel-A

FIG. 2A

Tyrosinase activity in cell extracts from Mel-A
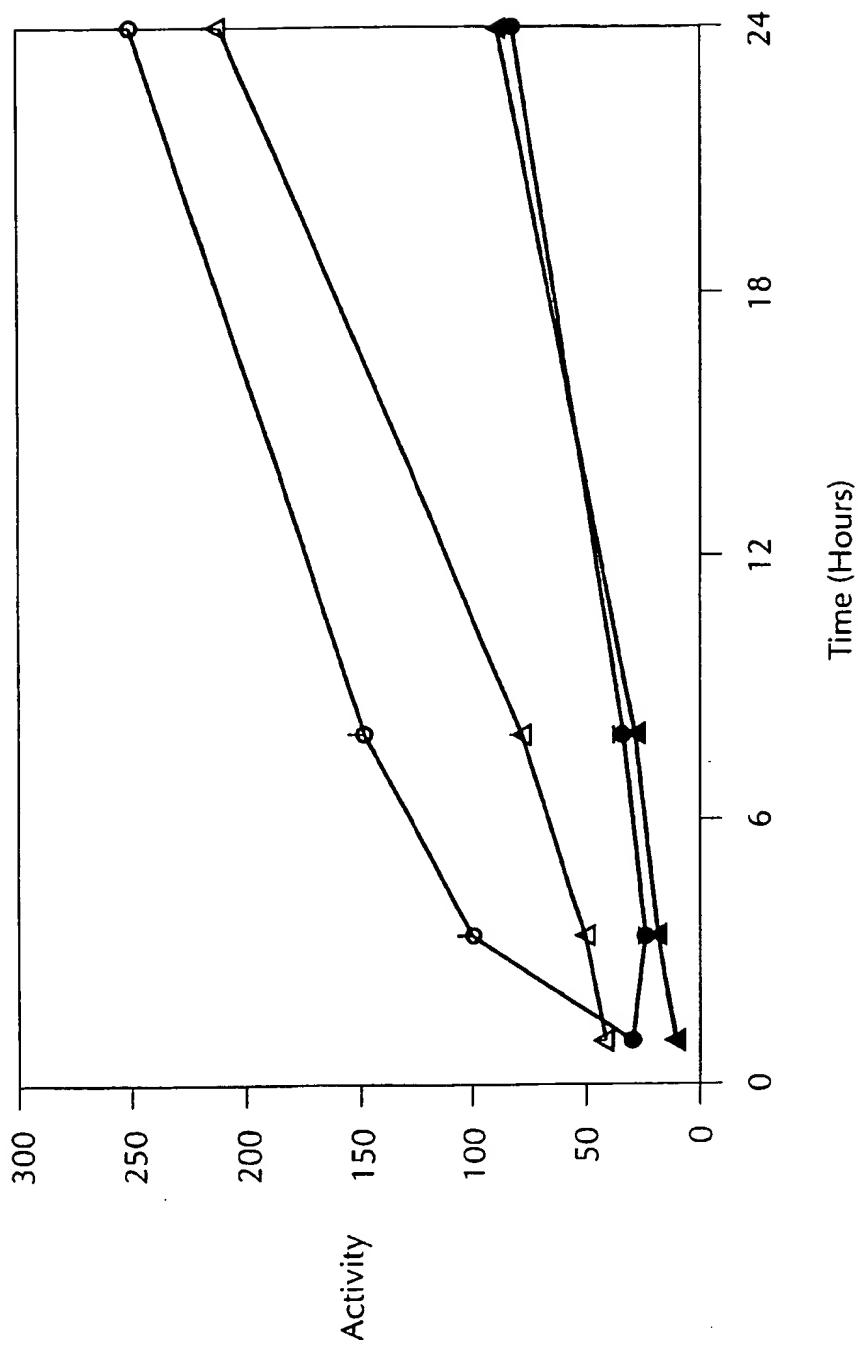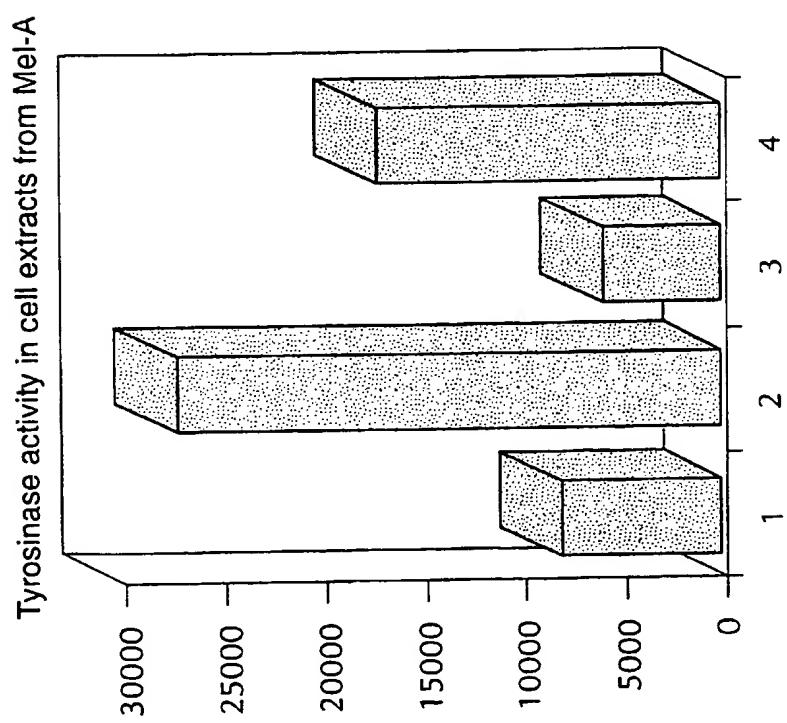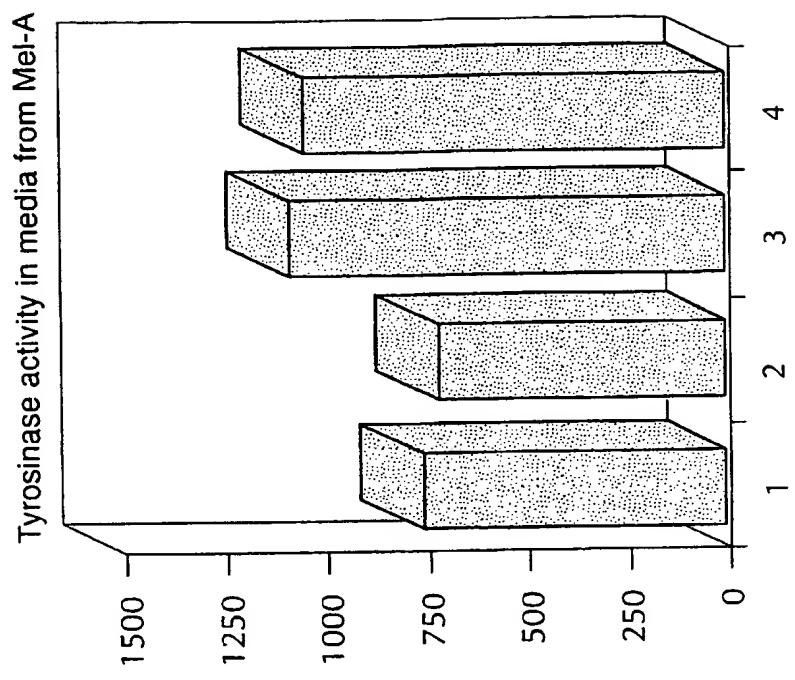
FIG. 3

Relative tyrosinase activity in transfected COS cells

4/13
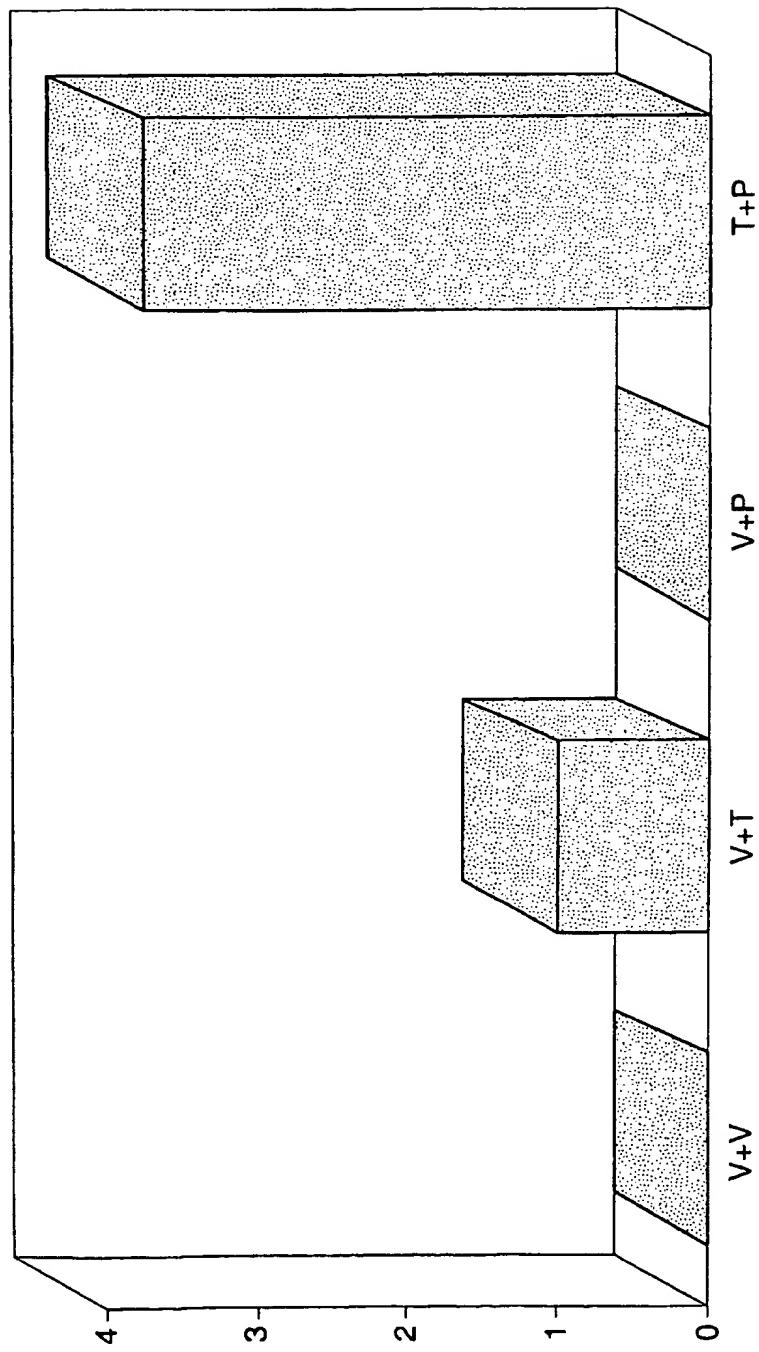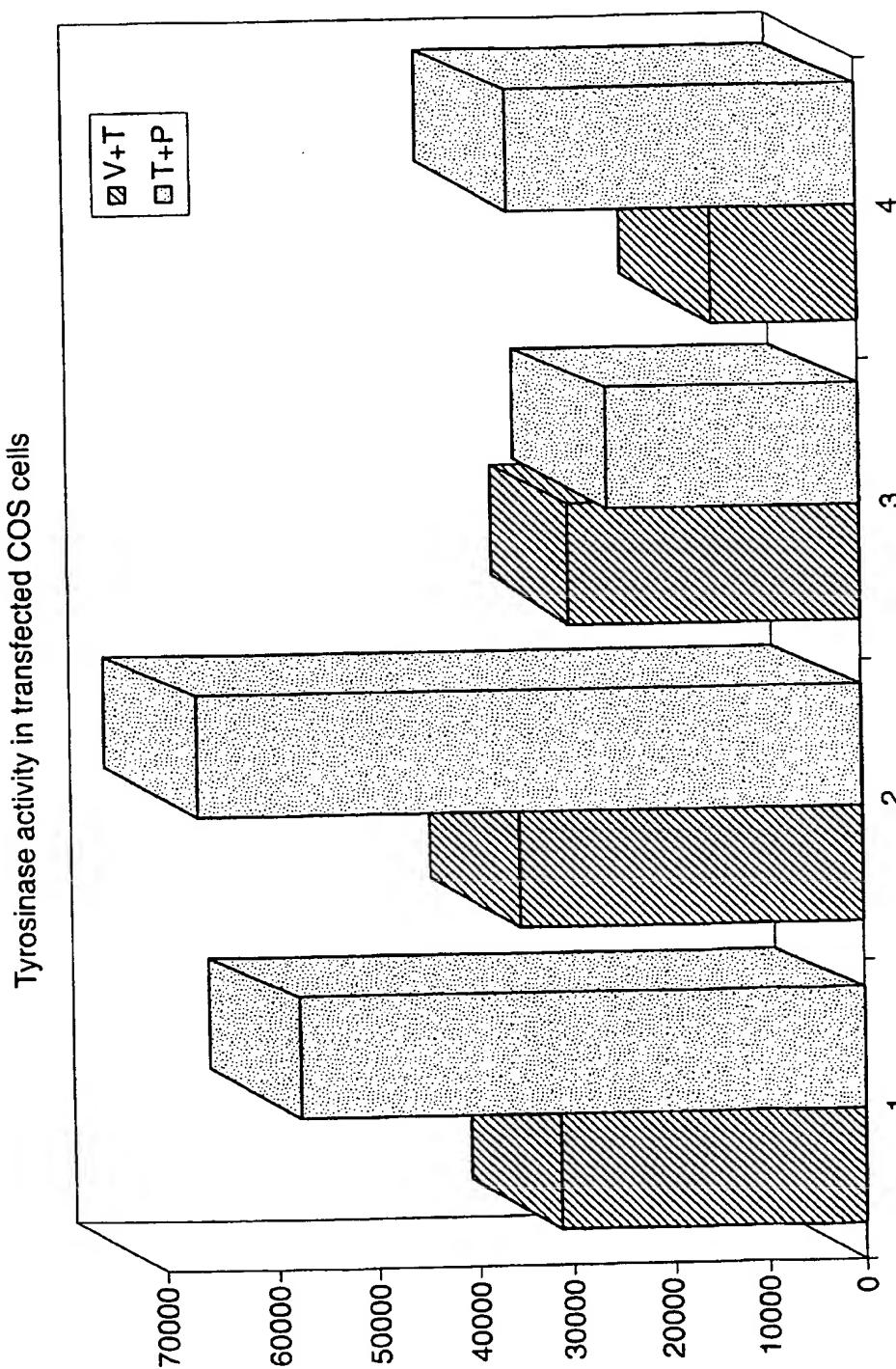
FIG. 4
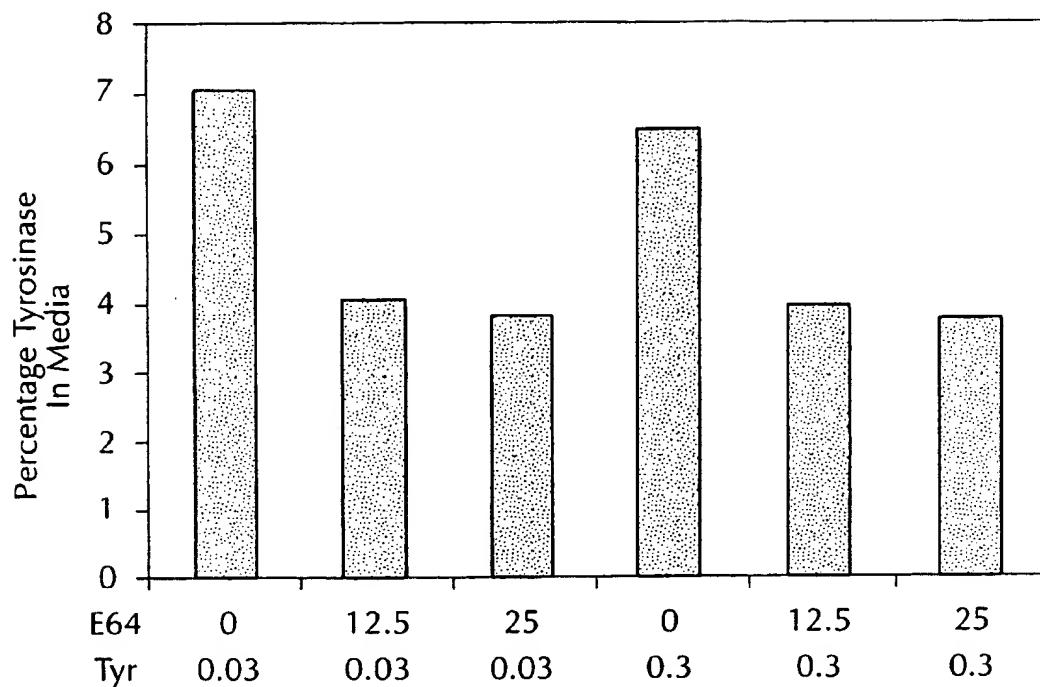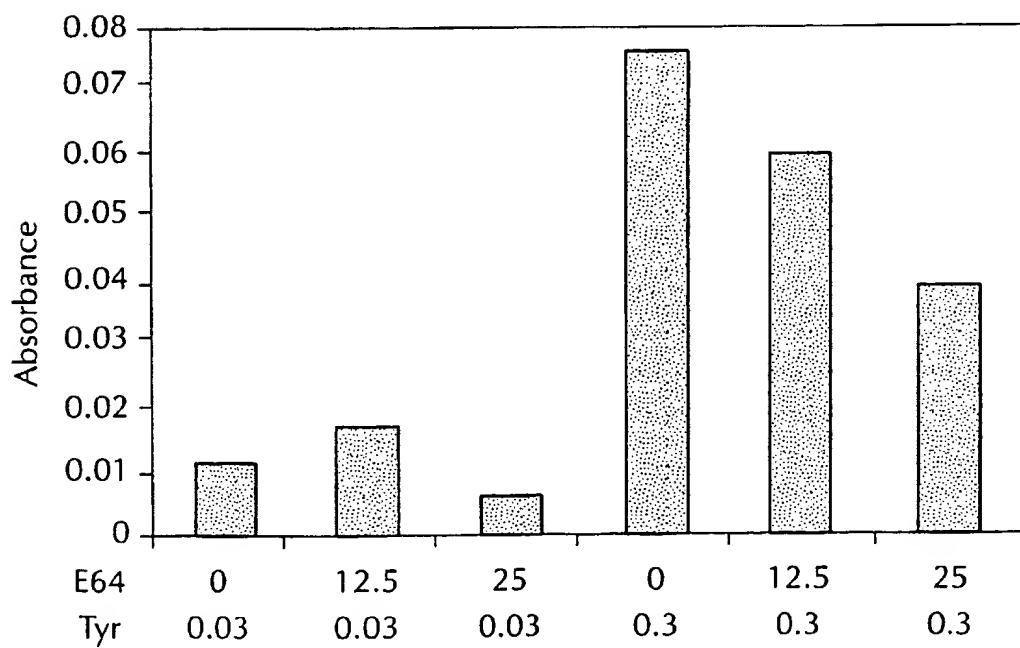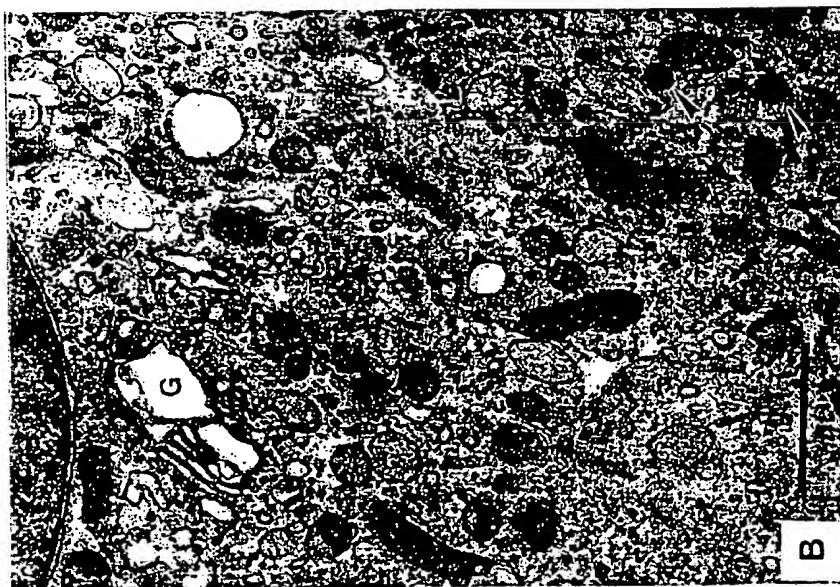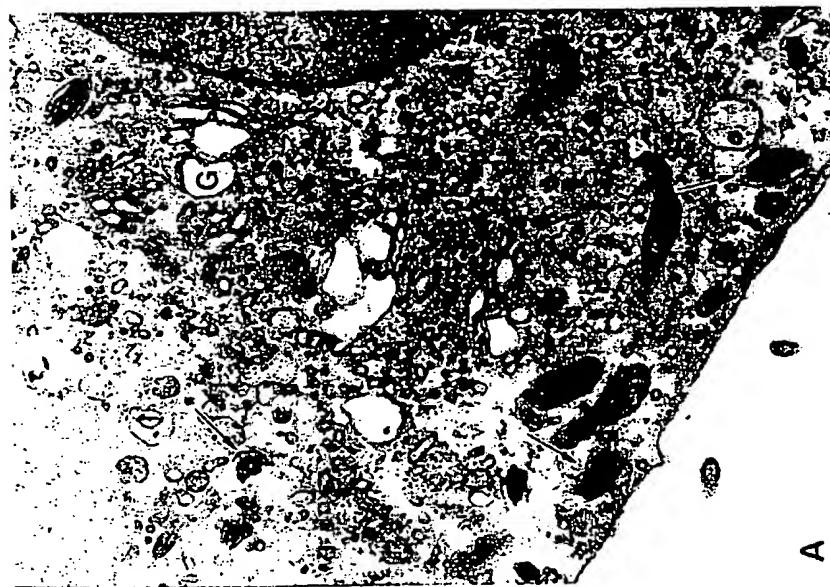
FIG. 5A

FIG. 5B

FIG. 6B

FIG. 6A

FIG. 7A

FIG. 7B

FIG. 8B
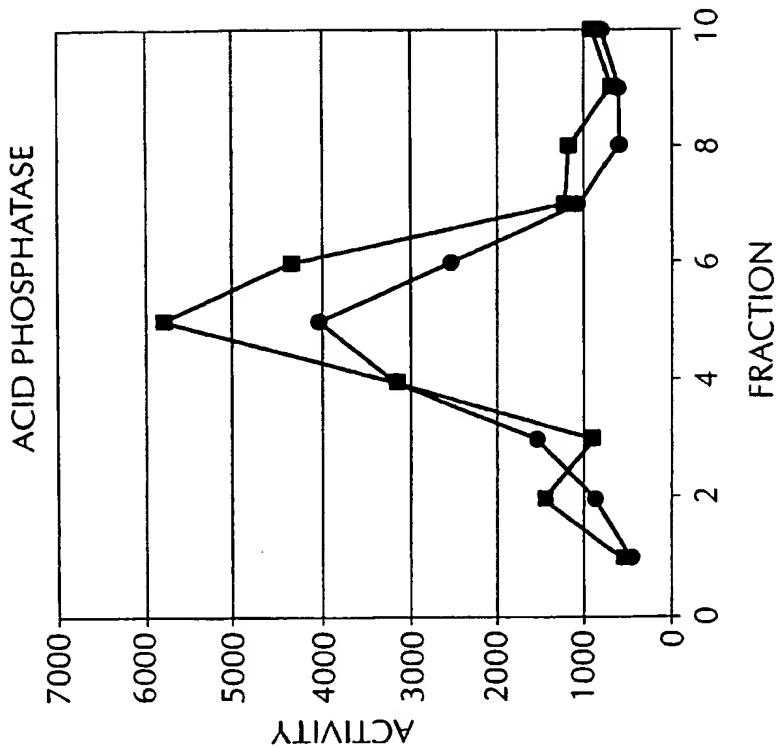
FIG. 8A
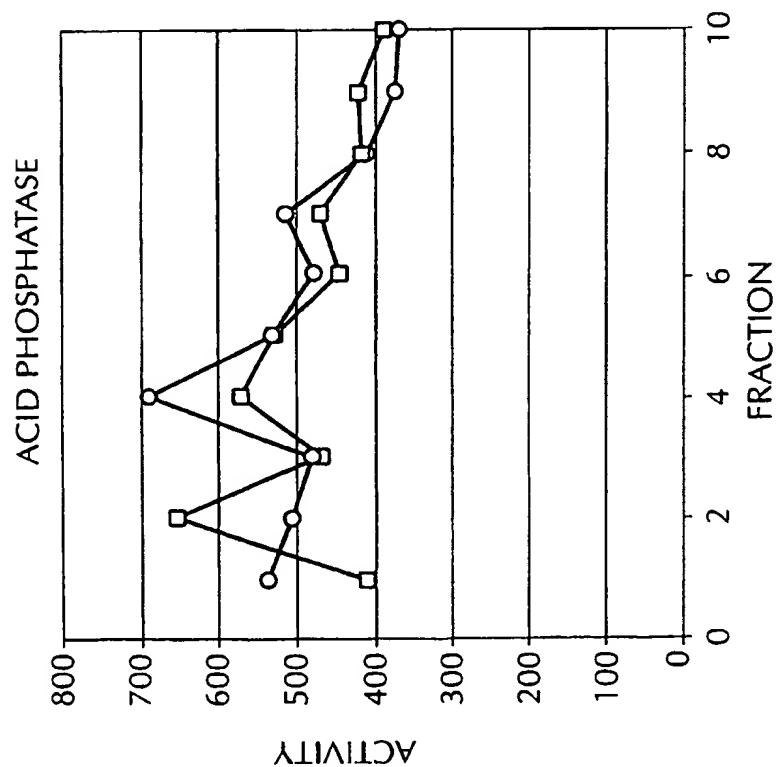
FIG. 9B

FIG. 9A
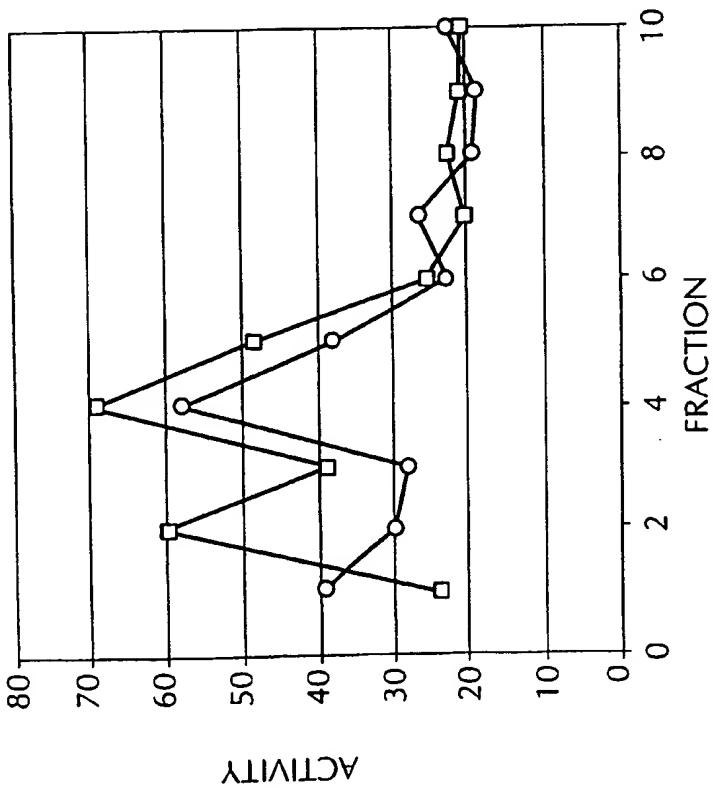
FIG. 10B
BETA-HEXOSAMINIDASE

FIG. 10A
BETA-HEXOSAMINIDASE

FIG. 11B
BETA-GLUCOSIDASE
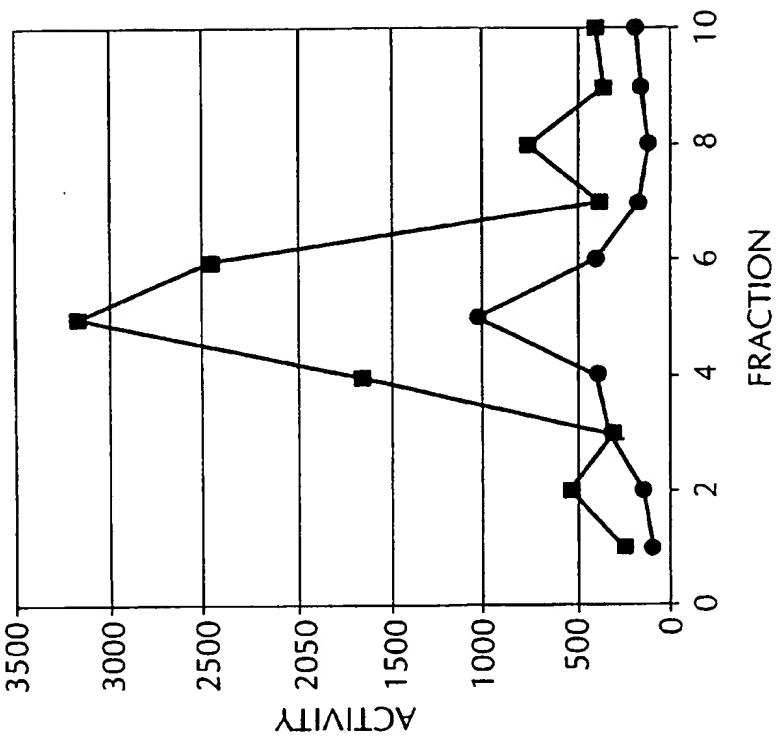
FIG. 11A
BETA-GLUCOSIDASE
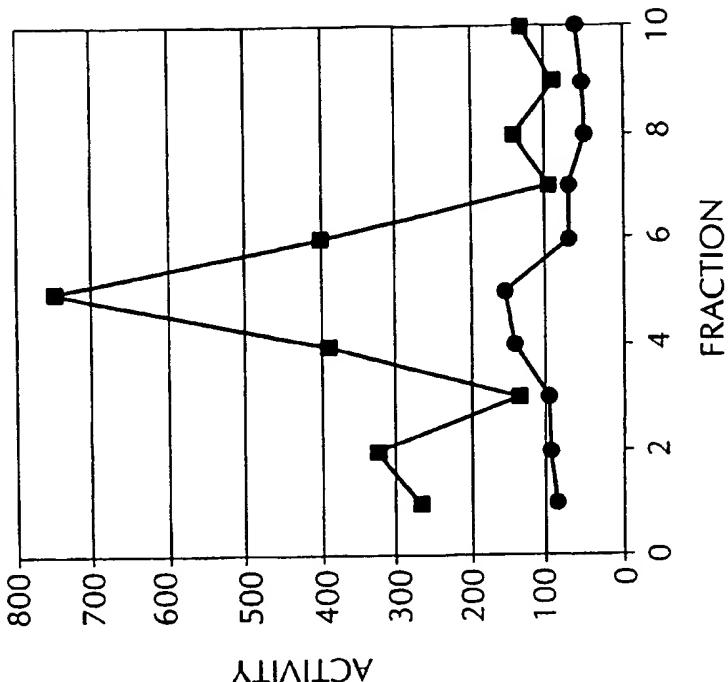
FIG. 12B

BETA-GLUCURONIDASE
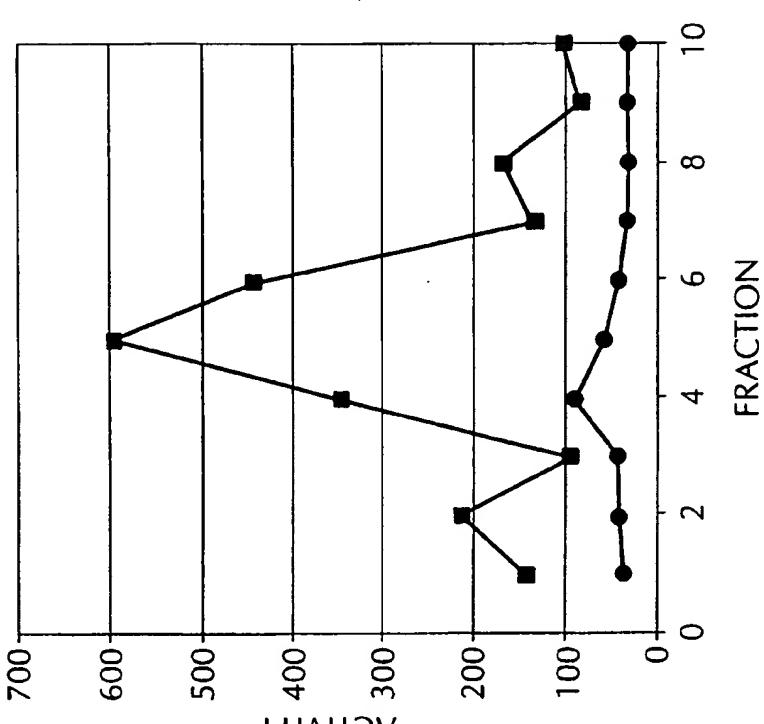
FIG. 12A

BETA-GLUCURONIDASE
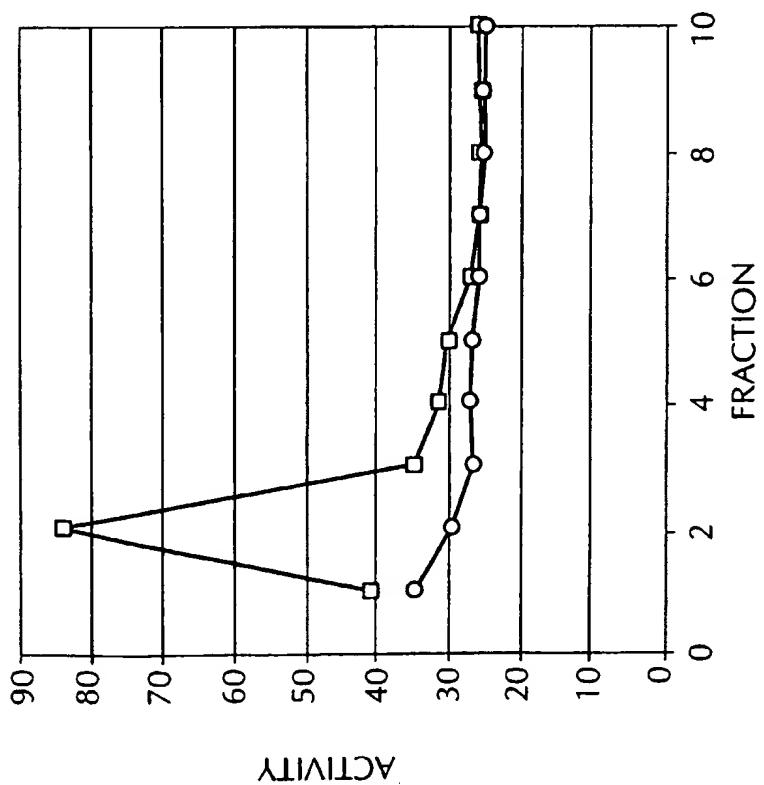
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   G01N33/50   G01N33/68   C12N9/02   C12Q1/26   A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   G01N   C12N   C12Q   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 04, 30 April 1996 (1996-04-30) & JP 07 324095 A (HIDEJI ITOKAWA ET AL.), 12 December 1995 (1995-12-12) abstract ---	1-17
A	US 5 628 987 A (FULLER, BRYAN B.) 13 May 1997 (1997-05-13) abstract; example 4 --- -/--	18-31

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 November 2000	15/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

Intern   ral Application No

PCT/IB 00/00861

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MEDLINE Washington DC USA; abstract no. 20133868, abstract XP002151889 & W.S. OETTING ET AL.: "Mutations of the human P gene associated with Type II oculocutaneous albinism (OCA2)" HUMAN MUTATION, vol. 12, no. 6, 1998, page 434 Minneapolis MN USA	32-43
A	CHEMICAL ABSTRACTS, vol. 126, no. 25, 23 June 1997 (1997-06-23) Columbus, Ohio, US; abstract no. 328544, XP002151890 abstract & D.N. HU ET AL.: "Regulation of melanogenesis by human uveal melanocytes in vitro" EXPERIMENTAL EYE RESEACH, vol. 64, no. 3, 1997, pages 397-404, New York NY USA	1-43

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
JP 07324095 A	12-12-1995	NONE		
US 5628987 A	13-05-1997	US	5540914 A	30-07-1996
		US	5554359 A	10-09-1996
		US	5589161 A	31-12-1996
		US	5591423 A	07-01-1997

Mitogenic, Melanogenic, and cAMP Responses of Cultured Neonatal Human Melanocytes to Commonly Used Mitogens

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The following studies have been undertaken to compare and correlate the effects
of 12-0-tetradecanoylphorbol acetate (TPA), basic fibroblast growth factor (bFGF),
cholera toxin (CT), and isobutyl methylxanthine (IBMX) on neonatal human me-
lanocyte (NHM) proliferation, tyrosinase activity, and cyclic adenosine mono-
phosphate (cAMP) concentration. NHM proliferated at a maximal rate in medium
containing 8 nM TPA, 200 ng/ml CT, and 10^{-4} M IBMX. TPA alone did not result
in optimal melanocyte proliferation, and, as previously shown, its mitogenic
effect was greatly enhanced by the addition of CT and IBMX individually or
concomitantly. Human recombinant (hr) bFGF could replace TPA in the NHM
growth medium. Maximal proliferation was achieved using 3 ng/ml hrbFGF, 20
ng/ml CT, and 10^{-4} M IBMX. The mitogenic effect of 1.2 ng/ml hrbFGF was
potentiated in the concomitant but not individual presence of CT and IBMX. TPA
alone in the absence of CT and IBMX caused a dose-dependent stimulation of
tyrosinase activity. Maximal tyrosinase activity was obtained in the presence of
0.8 nM TPA, 20 ng/ml CT, and 10^{-4} M IBMX. Unlike TPA, hrbFGF alone resulted
in inhibition of tyrosinase activity. In the presence of hrbFGF, tyrosinase activity
was potentiated by CT and IBMX, but not by CT alone. Neither TPA nor hrbFGF
alone could increase intracellular cAMP levels. The effects of CT and IBMX on
intracellular cAMP concentration were enhanced to a greater extent by TPA than
by hrbFGF. Under our experimental conditions, in the presence of hrbFGF, CT but
not IBMX resulted in a dose-dependent increase in cAMP concentration. Further
studies on NHM will be aimed at determining the exact role of protein kinase C
(PKC) in regulating proliferation and melanogenesis and the mechanism(s) acti-
vated by hrbFGF.

The ability to maintain epidermal human melano-
cytes in culture has enabled investigators to begin iden-
tifying factors that regulate human pigmentation.
Most of the studies on human melanocytes have focused
on the regulation of proliferation. The first growth me-
dium for human melanocytes was described by Eisinger
and Marko, and was based on the use of two specific
mitogens: the phorbol ester 12-0-tetradecanoylphorbol
acetate (TPA) and cholera toxin (CT) (Eisinger and
Marko, 1982). In this medium, the survival of melano-
cytes was shown to be dependent on the presence of
TPA, and the mitogenic effect of TPA was shown to be
enhanced by CT (Eisinger and Marko, 1982; Halaban
et al., 1986). Since then, this medium has been modified
extensively and TPA has been replaced by a variety of
mitogens, such as bovine hypothalamic extract, placen-
tal extract, factors produced by melanoma cells, astro-
cytoma cells and fibroblasts, and most recently by basic
fibroblast growth factor (bFGF) and leukotrienes C_4
and D_4 (Wilkins et al., 1985; Eisinger et al., 1985; Hal-
aban et al., 1987; Morelli et al., 1989). In addition to CT,
isobutyl methylxanthine (IBMX) was included in the
melanocyte growth medium and shown to enhance me-

lanocyte proliferation further (Halaban et al., 1986).
All of the above conditions allowed neonatal but not
adult human melanocytes to be maintained for a long-
term in culture. Recently it was reported that adult
melanocytes from normal donors as well as vitiligo pa-
tients can be successfully grown and propagated for a
long term in culture in a medium containing three es-
sential mitogens: TPA, FGF, and bovine pituitary ex-
tract (Medrano and Nordlund, 1990).

Only a few studies have attempted to investigate the
effects of commonly used mitogens on melanogenesis in
human melanocytes. It is known that TPA binds to
protein kinase C (Castagna et al., 1982). In addition to
its proliferative effects, TPA has been shown to stimu-
late tyrosinase activity in neonatal human melano-
cytes (Halaban et al., 1983). CT and IBMX are known
to increase intracellular cyclic adenosine monophos-
phate (cAMP) concentration by activating adenylate

cyclase and inhibiting phosphodiesterase, respectively (Enomoto and Gill, 1980; Russel and Pasean, 1974). Both factors are known to be melanogenic for murine melanoma cells (O'Keefe and Cuatrecasas, 1974; Abdel-Malek et al., 1989). However, their effect and that of bFGF on melanin formation by normal human melanocytes has not been determined. The ability of TPA, CT, and IBMX to support the growth of human melanocytes in long-term culture suggests that melanocyte proliferation is modulated by two signal transduction systems: the phosphoinositol/protein kinase C (PKC) and the cAMP/protein kinase A (PKA) systems. The direct role and the possible interaction of these two signal transduction systems in regulating proliferation and melanogenesis of human melanocytes still remain unclear.

One concern about the use of human melanocytes grown in the presence of the tumor promoter TPA, the nucleotide analog IBMX, and the neurotoxin CT is that these cells are refractory to the effects of melanogenic factors and may not be a functional equivalent of their in vivo counterpart. Our goal is to grow melanocytes under conditions that allow them to proliferate optimally without maximally stimulating melanogenesis. Defining the intracellular mechanisms that regulate proliferation, tyrosinase activity, and melanogenesis should help us identify the physiologic regulatory factors that would enable us to achieve this goal.

The results presented here describe in detail the effects of TPA, CT, IBMX, and bFGF on neonatal human melanocyte (NHM) proliferation and melanization. Also, the effects of these factors on cAMP and the involvement of cAMP in the regulation of melanocyte proliferation and melanization have been studied and are reported here.

MATERIALS AND METHODS
Materials

Ham's F-10 medium, 0.25% trypsin solution, and penicillin–streptomycin solution were obtained from Gibco Laboratories (Grand Island, NY). MCDB 153 medium was a generous gift from Dr. Steven Boyce, Shriners' Burns Institute, Cincinnati, OH, and was later purchased from Sigma Chemical Company (St. Louis, MO). CT, IBMX, TPA, human transferrin, α-tocopherol, insulin, activated charcoal, and Dowex 50W were purchased from Sigma Chemical Company (St. Louis, MO). Newborn calf serum and fetal calf serum were purchased from ICN-Flow Laboratories (Costa Mesa, CA). Human recombinant (hr) bFGF was obtained from Boehringer-Mannheim (Indianapolis, IN). L-[Ring 3',5',³H]tyrosine (specific activity 46.7 Ci/mmol) and ¹²⁵I-labeled cAMP radioimmunoassay kits were obtained from New England Nuclear (Boston, MA).

Culture of neonatal human melanocytes from newborn foreskins

Foreskins were collected individually in sterile bottles containing MEM-S medium and processed immediately as follows. Ventral fatty tissues were trimmed and the foreskin was incubated in 0.25% trypsin solution overnight at 4°C. The epidermis was separated from the dermis, and both layers were transferred into a centrifuge tube containing melanocyte growth medium (described below) and vigorously vortexed. The resulting cell suspension was transferred into a 25 cm² tissue culture flask and incubated in a humidified incubator containing 5% CO_2 at 37°C.

Melanocytes were grown in NHM medium or in the modified NHM medium. NHM medium consisted of Ham's F-10 medium + 5% fetal calf serum (FCS) + 5% new born calf serum (NBCS) + 5 μg/ml insulin, 2 μg/ml transferrin, 2 μg/ml α-tocopherol, 1% penicillin/streptomycin, 10^{-3} M IBMX, 20 ng/ml CT, 8 nM TPA. The modified NHM medium consisted of MCDB 153 medium containing 1.25% FCS, 1.25% NBCS + 5 μg/ml insulin, 2 μg/ml transferrin, 2 μg/ml α-tocopherol, 1% penicillin/streptomycin, 10^{-3} M IBMX, 20 ng/ml CT, 1.2 ng/ml hrbFGF. Insulin, transferrin, and α-tocopherol have been shown to be useful for the maintenance of adult human melanocytes in long-term culture (Medrano and Nordlund, 1990).

We have found it necessary to replace Ham's F-10 medium with MCDB-153 medium in order to obtain an optimal mitogenic effect of hrbFGF. When adding hrbFGF to Ham's F-10, melanocytes often failed to proliferate and could not be maintained for a prolonged time period in culture. MCDB-153 medium is commonly used for growing keratinocytes in culture, primarily due to its low calcium content (0.342 mg $CaCl_2$/l MCDB-153 compared to 4.411 mg $CaCl_2$/l F-10), a requirement for keratinocyte proliferation (Hennings et al., 1980). MCDB-153 medium differs from F-10 medium in several aspects, e.g., it is richer than F-10 medium in L-glutamine, proline, serine, threonine, choline chloride, inositol, and putrescine. The possible involvement of some or all of these constituents and calcium in establishing permissive conditions for hrbFGF action is not known.

The two melanocyte growth media described above selectively supported melanocyte proliferation, and keratinocytes did not persist after the first passage. Fibroblasts were eliminated from the cultures by treatment with 100 μg/ml Geneticin as described by Halaban and Alfano (1984).

Effects of mitogens on tyrosinase activity and proliferation

To determine the effects of various mitogens on proliferation and tyrosinase activity, melanocytes were seeded into 24 cluster wells (2.0 cm² surface area) at a density of 0.5×10^5 cells/well, or into 6 cluster wells (9.6 cm² surface area) at a density of $0.9–1.3 \times 10^5$ cells/well. The appropriate treatment was added to the culture wells 24 hours after seeding, and every other day thereafter for 6–7 days unless otherwise specified. Twenty-four hours prior to ending the experiment, medium containing ³H-tyrosine at a concentration of 1 μCi/ml was added to all cultures. At the end of each experiment, the conditioned medium from each culture well was collected and assayed directly, or stored at 0–4°C to be assayed later for tyrosinase activity. The cells in each well were harvested and counted using a Coulter counter.

Tyrosinase assay. The ³H-labeled conditioned medium was assayed according to the modified charcoal absorption method of Pomerantz (1969) as previously described (Fuller and Viskochil, 1979; Abdel-Malek

418

et al., 1989). The assay is based on measuring the amount of ^3H_2O released into the medium as tyrosine is converted to L-DOPA, a reaction catalyzed by the tyrosine hydroxylase activity of tyrosinase. Tyrosinase activity was expressed as counts/min/10^6 cells and as percent of control.

Determination of cAMP concentration

Melanocytes were seeded at a density of 5 × 10^5 cells/100 mm diameter Petri dish or at a density of 2.5 × 10^6 cells/60 mm diameter Petri dish. The cultures were then subjected to the experimental conditions starting 24 hours after seeding with fresh medium added every other day for 6 days, as described earlier for other experiments, unless otherwise stated. At the end of each experiment, cells were harvested by gentle scraping with a rubber policeman, counted, centrifuged, and then resuspended in 6% trichloroacetic acid for 1.5–2 hours at 4°C. The cells were centrifuged for 15 minutes, and the supernatant from each sample was collected and extracted four times with 5× volume of water-saturated ether, with the ether phase discarded each time. The aqueous phase from each sample was then dried on a hot plate at 70–80°C under a stream of pure nitrogen gas. The samples were resuspended in cold assay buffer (sodium acetate, pH 6.2, supplied in the radioimmunoassay kit) containing 1 mM theophylline. The volume of the assay buffer for each sample was adjusted to yield 0.7–0.8 × 10^5 cells extracted/100 μl buffer. Duplicate or triplicate 100 μl aliquots were taken from each sample to be assayed for cAMP as described in the radioimmunoassay kit.

RESULTS
Dose-dependent effect of TPA on NHM proliferation and the potentiation of this effect by CT and IBMX

In order to determine the effects of TPA and its interaction with CT and IBMX, a series of dose-response experiments have been carried out. Our results show that the proliferative response of NHM to TPA was much greater in the presence of 20 ng/ml CT and 10^{-4} M IBMX than in the total absence of these two agents, at the three concentrations of TPA tested: 0.8, 8, and 80 nM (Fig. 1). In the concomitant presence of CT and IBMX, NHM demonstrated a dose-dependent increase in proliferation in response to TPA. Under these conditions, the cell number achieved at the end of the experiment in medium containing 8 or 80 nM TPA was 2.5- and 3-fold higher than that caused by 0.8 nM TPA. When deprived of CT and IBMX for 7–8 days, NHM survived, but they did not proliferate in the presence of 0.8 nM TPA alone and replicated only once in the presence of 8 or 80 nM TPA (Fig. 1).

The contribution of CT and IBMX to the mitogenic effect of TPA was determined by adding either CT or IBMX to a medium containing 8 nM TPA (Table 1). The addition of 10^{-4} M IBMX resulted in a 64% increase while the addition of 20 ng/ml CT contributed a 49% increase in NHM proliferation (Table 1, experiment I). In medium containing 8 nM TPA and 20 ng/ml CT, the addition of IBMX caused a dose-dependent stimulation of proliferation. With the addition of IBMX at 10^{-6}, 10^{-5}, and 10^{-4} M, the cell number increased by 61%,
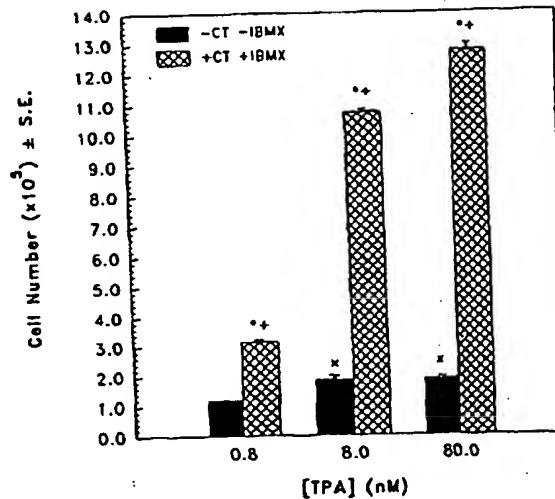
Fig. 1. The mitogenic effect of 12-O-tetradecanoylphorbol acetate (TPA) on NHM in the absence or presence of CT and IBMX. Neonatal human melanocytes were seeded onto 6 cluster wells at a density of 1.3 × 10^5 cells/well. Triplicate wells were seeded for each experimental group. The following day and every other day thereafter, each experimental group received the appropriate concentration of TPA in the absence or presence of 20 ng/ml cholera toxin (CT) and 10^{-4} M isobutyl methylxanthine (IBMX) for a total of 7 days. At the end of each experiment, cells from each well were detached and counted using a Coulter Counter Model ZM. This experiment was repeated four times with four different cell strains and similar results were obtained consistently. *, Each of 0.8, 8.0, and 80 nM TPA, +CT +IBMX is statistically different from its respective group −CT −IBMX at $P \le 0.05$. +, Groups are statistically different from each other at $P \le 0.05$. x, Groups are statistically different from 0.8 nM TPA −CT −IBMX but not different from each other at $P \le 0.05$.

74%, and 100% above the cell number obtained under control conditions (i.e., −CT, −IBMX, Table 1, experiment I).

The mitogenic effect of CT was also investigated by adding 2, 20, or 200 ng/ml CT to a medium containing 10^{-4} M IBMX and 8 nM TPA (Table 1, experiment II). Melanocytes proliferated at approximately the same rate in response to 2 and 20 ng/ml CT in their medium but had a significantly higher proliferative rate in response to 200 ng/ml CT. At this latter concentration, the cell density achieved at the end of the experiment was 35% and 26% greater than that obtained in the presence of 2 or 20 ng/ml CT, respectively.

From these results, it can be concluded that melanocytes respond in a dose-dependent manner to TPA, and that this response is greatly potentiated by CT and IBMX (Fig. 1, Table 1). Maximal proliferation can be achieved in the presence of 8 nM TPA, 200 ng/ml CT, and 10^{-4} M IBMX (Table 1, experiment II).

Dose-dependent effects of hrbFGF, CT, and IBMX on NHM proliferation

As observed with TPA, in medium containing hrbFGF, NHM proliferated at a faster rate in the presence than in the absence of 20 ng/ml CT and 10^{-4} M IBMX (Fig. 2). The mitogenic effect of hrbFGF was

TABLE 1. Dose response of NHM grown in the presence of 8 nM TPA to CT and IBMX[1]

	Cellular proliferation		Tyrosinase activity	
	Cell no. x 10^5	% of control	cpm/10^6 cells	% of control
Experiment I				
-CT, -IBMX (control)	0.66 ± 0.02	100 ± 3	21,047 ± 816	100 ± 4
-CT, +10^-4 M IBMX	1.08 ± 0.06	164 ± 9*	37,294 ± 2,840	177 ± 14*
+CT, -IBMX	0.98 ± 0.02	149 ± 3*	52,135 ± 372	248 ± 18*,**
+CT, +10^-6 M IBMX	1.06 ± 0.06	161 ± 9*	69,532 ± 4,938	330 ± 24*,**
+CT, +10^-5 M IBMX	1.15 ± 0.10	174 ± 16*	77,075 ± 777	366 ± 4*,**
+CT, +10^-4 M IBMX	1.32 ± 0.06	200 ± 9*,**,***·†	65,270 ± 3,547	310 ± 17*,**
Experiment II				
IBMX, +2 ng/ml CT	1.52 ± 0.15	100 ± 10	61,465 ± 2,414	100 ± 4
IBMX, +20 ng/ml CT	1.63 ± 0.07	107 ± 5	54,807 ± 3,369	89 ± 6
IBMX, +200 ng/ml CT	2.05 ± 0.06	135 ± 4††·†††	72,031 ± 3,652	117 ± 6†††

[1] Melanocytes were seeded at an initial density of 0.5 x 10^5 cells in 2 cm^2 wells. Twenty-four hours later the appropriate experimental media were added and the media were changed every other day thereafter for a total of 7 days. In experiment I, all media contained 8 nM TPA and 20 ng/ml CT unless mentioned otherwise. In experiment II, all media contained 8 nM TPA + 10^-4 M IBMX, and the cell strain used was different from that used in experiment I. n = 3 for cellular proliferation and n = 6 for tyrosinase activity. The above results are those of an experiment that was repeated twice with similar findings. Values are ± SE. CT, cholera toxin; IBMX, isobutyl methylxanthine; NHM, neonatal human melanocyte; TPA, 12-O-tetradecanoylphorbol acetate.
* Values are statistically different from the -CT, -IBMX treatment group, $P \leq 0.05$.
** Values are statistically different from -CT, +10^-4 M IBMX treatment group, $P \leq 0.05$.
*** Values are statistically different from +CT, +10^-6 M IBMX at $P \leq 0.05$.
† Values are statistically different from +CT, -IBMX treatment group at $P \leq 0.05$.
†† Values are statistically different from IBMX, +2 ng/ml CT treatment group, $P \leq 0.05$.
††† Values are statistically different from IBMX, +20 ng/ml CT treatment group, $P \leq 0.05$.

greatly potentiated (two- to fourfold) by the addition of 20 ng/ml CT and 10^-4 M IBMX to a medium containing 0.6, 1.2, 3, or 6 ng/ml hrbFGF, and maximal proliferation was obtained in response to 3 ng/ml hrbFGF. In medium lacking CT and IBMX, increasing the concentration of hrbFGF from 0.6 to 1.2, 3, and 6 ng/ml resulted in a minimal increase in cellular proliferation. Under these conditions, NHM demonstrated a 30–40% increase in proliferation in the presence of 6 ng/ml hrbFGF over that obtained in the presence of 0.6 ng/ml (Fig. 2).

In the presence of 1.2 ng/ml hrbFGF and 20 ng/ml CT, NHM responded to increasing concentrations of IBMX with a dose-dependent increase in proliferation. Under these conditions, 10^-5 M IBMX resulted in a maximal increase in cell number, which was 161% greater than that obtained in the absence of IBMX or of IBMX and CT (Table 2, experiment I).

In medium containing 1.2 ng/ml hrbFGF and 10^-4 M IBMX, NHM demonstrated a gradual and significant increase in proliferation in response to increasing concentrations of CT. The number of NHM obtained in the presence of 20 ng/ml CT was 70% higher than that achieved in the absence of CT alone or of both CT and IBMX, and 46% higher than that caused by 2 ng/ml CT (Table 2, experiment II). Increasing the concentration of CT from 20 to 200 ng/ml CT did not further potentiate proliferation. An interesting observation is that in medium containing hrbFGF, the presence of both CT and IBMX was obligatory for the enhancement of the mitogenic effect of hrbFGF. Neither CT nor IBMX alone increased proliferation above that achieved under control conditions (i.e., -CT, -IBMX, Table 2, experiments I and II).

These results confirm previous findings that hrbFGF can substitute for TPA in the NHM growth medium (Halaban et al., 1987). However, unlike TPA whose mitogenic effect on NHM can be potentiated by either CT or IBMX, hrbFGF requires the concomitant pres-

ence of both of these factors for synergistic stimulation of NHM proliferation.

Dose-dependent effects of TPA, CT, and IBMX on tyrosinase activity

In NHM medium lacking CT and IBMX, increasing the concentration of TPA from 0.8 to 8 nM resulted in a 1.5-fold increase in tyrosinase activity (Fig. 3). No further stimulation of tyrosinase activity was obtained as the concentration of TPA was further increased to 80 nM. In the presence of 20 ng/ml CT and 10^-4 M IBMX, the activity of tyrosinase was greatly potentiated at the three concentrations of TPA tested (0.8, 8, and 80 nM), and was maximal in the presence of 0.8 nM (Fig. 3). The potentiating effects of CT and IBMX gradually diminished as the concentration of TPA was increased to 8 and 80 nM.

In the presence of 8 nM TPA and 20 ng/ml CT, maximal tyrosinase activity was achieved at 10^-6 M IBMX and was 230% above control and 33% above the activity measured in the absence of IBMX (Table 1, experiment I). In the absence of CT, 10^-4 M IBMX contributed a 77% increase in tyrosinase activity above control level. In comparison, 20 ng/ml CT enhanced tyrosinase activity by 148% above control, in the absence of IBMX (Table 1, experiment I). The use of 2 or 20 ng/ml CT, in the concomitant presence of 10^-4 M IBMX, had a comparable effect on tyrosinase activity. This effect, however, was significantly enhanced as the concentration of CT was further increased to 200 ng/ml (Table 1, experiment II).

Dose-dependent effects of hrbFGF, CT, and IBMX on tyrosinase activity

We have found that hrbFGF at concentrations ranging from 0.6 to 6 ng/ml decreased tyrosinase activity in a dose-dependent fashion. This effect was observed in the absence and in the presence of CT and IBMX (Fig. 4). In the absence of these two agents, tyrosinase
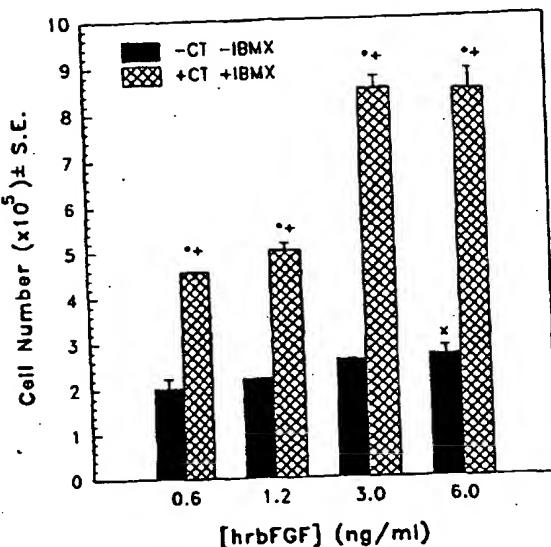
Fig. 2. The mitogenic effect of human recombinant basic fibroblast growth factor (hrbFGF) on NHM in the absence or presence of cholera toxin (CT) and isobutyl methylxanthine (IBMX). Neonatal human melanocytes grown in medium containing 1.2 ng/ml hrbFGF, 20 ng/ml CT, and 10^{-4} M IBMX were seeded onto 6 cluster wells at a density of 1.0×10^5 cells/well. On the following day and every other day thereafter for 7 days, each experimental group received medium containing the appropriate concentration of hrbFGF, in the absence or presence of 20 ng/ml CT and 10^{-4} M IBMX. For each group, n = 3. This experiment was repeated five times using five different cell strains. *, Each concentration of hrbFGF +CT +IBMX is statistically different from its −CT −IBMX counterpart at $P \leq 0.05$. +, 0.6, 1.2, and 3 ng/ml hrbFGF each in the presence of CT and IBMX are statistically different from each other. 6 ng/ml hrbFGF is statistically different from 0.6 and 1.2 ng/ml hrbFGF, but not from 3 ng/ml, in the presence of CT and IBMX, at $P \leq 0.05$. x, Values are statistically different from 0.6 ng/ml hrbFGF −CT −IBMX at $P \leq 0.05$.

activity was either unaffected (two of five cell strains tested) or diminished (three of five strains) by increasing concentrations of hrbFGF. As expected, tyrosinase activity was greater in the presence than in the absence of CT and IBMX at 1.2 and 3 ng/ml hrbFGF.

A dose-dependent stimulation of tyrosinase activity was obtained in response to IBMX and CT (Table 2). Maximal tyrosinase activity was measured in response to 10^{-4} IBMX in medium containing 1.2 ng/ml hrbFGF and 20 ng/ml CT (Table 2, experiment I). Under these conditions, tyrosinase activity increased 86% and 116% above that measured in the absence of IBMX or under control conditions, respectively. In the presence of 10^{-4} M IBMX and following the addition of 2, 20, and 200 ng/ml CT, tyrosinase activity was elevated by 21%, 53%, and 59%, respectively, above that measured in the absence of CT (Table 2, experiment II). Interestingly, in the absence of IBMX, CT at 20 ng/ml had no significant stimulatory effect on tyrosinase (Table 2, experiment I). However, in the absence of CT, 10^{-4} M IBMX elicited a 122% stimulation of tyrosinase activity above control (Table 2, experiment II).

These experiments suggest that while hrbFGF is mitogenic to NHM, it decreases tyrosinase activity. The addition of both CT and IBMX to the hrbFGF-containing medium results in a higher tyrosinase activity than that obtained in the absence of CT and IBMX (Fig. 4, Table 2). IBMX in the absence of CT can stimulate tyrosinase activity. However, the stimulatory effect of CT is only evident in the concomitant presence of IBMX (Table 2).

Morphology of NHM grown in medium containing hrbFGF

It is known that NHM grown in the presence of TPA are uniformly bipolar (Fig. 5A). However, in the presence of hrbFGF concomitantly with CT and IBMX, NHM had a heterogeneous morphology. Some were either epithelioid or bipolar, and some others were polydendritic (Fig. 5B). The dendricity of the melanocytes was increased by increasing the concentration of hrbFGF in the culture medium. High concentrations of hrbFGF (≥ 3 ng/ml) caused the appearance of many polydendritic melanocytes (Fig. 5C). The morphology of NHM was greatly modified by depleting the hrbFGF containing medium of CT and IBMX. The absence of these two factors produced a very uniform morphologic pattern. All NHM became more epithelioid and bipolar with short dendrites (Fig. 5D). This change in morphology was evident 48 hours after removal of CT and IBMX from the growth medium containing hrbFGF and was not observed in cultures maintained in the presence of TPA.

Induction of cyclic AMP by TPA, CT, and IBMX

In the presence of 20 ng/ml CT and 10^{-4} M IBMX, TPA increased cAMP concentration dose-dependently. Increasing the concentration of TPA from 0.8 to 8 and 80 nM in the presence of the above concentrations of CT and IBMX resulted in a 67% and a 223% elevation of cAMP concentration (Table 3, experiment I). In medium lacking CT and IBMX, increasing the concentration of TPA from 0.8 to 8 and 80 nM had no significant effect on cAMP concentration (data not shown).

As expected, both IBMX and CT increased cAMP concentration in a dose-dependent fashion (Table 4). IBMX alone at 10^{-4} M contributed a 42% increase in cAMP level above that measured in the absence of CT and IBMX. The concomitant presence of 20 ng/ml CT and 10^{-5} or 10^{-4} M IBMX caused NHM to express a 63% and a 250% increase in cAMP concentration, respectively, above that measured in the control group. CT at 20 ng/ml, in the absence of IBMX, resulted in a 17% higher cAMP level than that measured in the control group. An 85% increase in cAMP level above control resulted from the concomitant addition of 2 ng/ml CT and 10^{-4} M IBMX. This increase was potentiated by 90% as the concentration of CT was increased from 2 to 20 ng/ml, but was not further enhanced by 200 ng/ml CT (Table 4).

The dose-dependent effect of hrbFGF on cAMP concentration in the absence or presence of CT and IBMX was investigated (Table 3, experiment II). In the absence of CT and IBMX, NHM grown for 7 days in medium containing 3 ng/ml hrbFGF expressed a minimal increase in cAMP concentration above that measured in NHM grown in medium containing 1.2 ng/ml hrbFGF (data not shown). This increase, although small, was consistently observed in three experiments using three different cell strains, and was not further

TABLE 2. Dose response of NHM grown in the presence of 1.2 ng/ml hrbFGF to IBMX and CT[1]

	Cellular proliferation		Tyrosinase activity	
	Cell no. $\times 10^5$	% of control	cpm/10^6 cells	% of control
Experiment I				
−CT, −IBMX (control)	1.53 ± 0.011	100 ± 1	20,009 ± 871	100 ± 4
+CT, −IBMX	1.41 ± 0.02	92 ± 1	23,297 ± 1,961	116 ± 10
+CT, +10^{-6} M IBMX	1.56 ± 0.03	102 ± 2	24,487 ± 1,063	122 ± 5*
+CT, +10^{-5} M IBMX	4.00 ± 0.40	261 ± 26*,**	25,928 ± 526	130 ± 3*
+CT, +10^{-4} M IBMX	4.37 ± 0.60	286 ± 39*,**	43,243 ± 1,433	216 ± 7*,**
Experiment II				
−CT, −IBMX (control)	3.96 ± 0.21	100 ± 5	27,756 ± 1,471	100 ± 5
+IBMX, −CT	4.07 ± 0.06	103 ± 2	61,475 ± 1,177	222 ± 4***
+IBMX, +2 ng/ml CT	4.68 ± 0.07	118 ± 2***,†	74,572 ± 1,501	269 ± 5***,†
+IBMX, +20 ng/ml CT	6.83 ± 0.38	173 ± 10***,†,††	94,097 ± 3,085	339 ± 11***,†,††
+IBMX, +200 ng/ml CT	6.13 ± 0.08	155 ± 2***,†,††	97,709 ± 1,791	352 ± 7***,†,††

[1]NHM from two different donors were seeded for the above two experiments at a density of 0.9 × 10⁵ cells/well and exposed the following day to the above experimental conditions. The medium was changed every other day for a total of 7 days. In experiment I, all groups had 20 ng/ml CT, except for the control. In experiment II, all groups had 10^{-4} M IBMX, except for the control. For each group n = 3 for cell number and n = 6 for tyrosinase activity. Values are ± SE. hrbFGF, human recombinant basic fibroblast growth factor. For other abbreviations, see footnote 1 to Table 1.
* Values are statistically different from the −CT and −IBMX treatment group, $P \leq 0.05$.
** Values are statistically different from the +CT and −IBMX treatment group, $P \leq 0.05$.
*** Values are statistically different from −CT and −IBMX treatment group, $P \leq 0.05$.
† Values are statistically different from +IBMX and −CT treatment group, $P \leq 0.05$.
†† Values are statistically different from +IBMX, +2 ng/ml CT treatment group but not different from each other, $P \leq 0.05$.
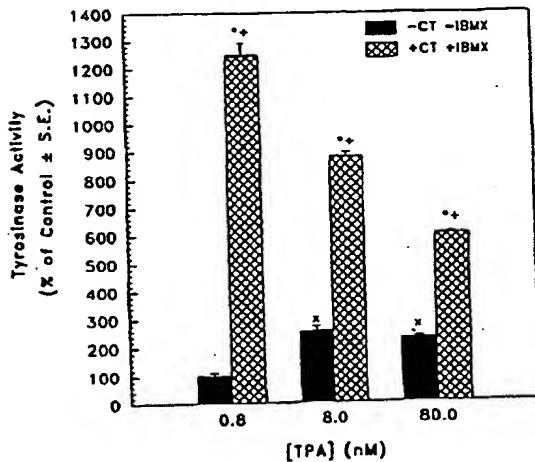
Fig. 3. The effect of 12-O-tetradecanoylphorbol acetate (TPA) on tyrosinase activity of NHM maintained in the absence or presence of cholera toxin (CT) and isobutyl methylxanthine (IBMX). At the end of the experiment presented in Figure 1, tyrosinase activity was determined as described in Materials and Methods. For each group, n = 6. Similar results were obtained with four different cell strains tested. *, Values are statistically different from control (0.8 nM TPA −CT −IBMX) at each concentration of TPA at $P \leq 0.05$. +, Groups representing 0.8, 8.0, and 80 nM TPA +CT +IBMX are statistically different from each other at $P \leq 0.05$. x, Values are statistically different from 0.8 nM TPA −CT −IBMX but not from each other at $P \leq 0.05$.

Fig. 4. Dose-dependent effect of human recombinant basic fibroblast growth factor (hrbFGF) on tyrosinase activity, in the absence or presence of cholera-toxin (CT) and isobutyl methylxanthine (IBMX). Melanocytes were seeded at 1.3 × 10⁵ cells/well and exposed to the above experimental conditions for a total of 6 days as described in Materials and Methods. In each group n = 6. This experiment was repeated five times using five different cell strains. The control in this experiment is the group maintained in the presence of 1.2 ng/ml hrbFGF −CT −IBMX. *, Each group +CT +IBMX is statistically different from its −CT −IBMX counterpart at each concentration of hrbFGF tested, at $P \leq 0.05$. +, Groups representing 1.2, 3.0, and 6.0 ng/ml hrbFGF +CT and IBMX are statistically different from each other at $P \leq 0.05$. x, Groups are statistically different from control but not from each other at $P \leq 0.05$.

enhanced by increasing the concentration of hrbFGF to 6 ng/ml. In the presence of CT and IBMX, the hrbFGF-induced cAMP concentration was greater in the presence than in the absence of CT and IBMX and was slightly increased when the concentration of hrbFGF was raised to 6 ng/ml (Table 3, experiment II).

In medium containing 1.2 ng/ml hrbFGF and 20 ng/ml CT, the greatest increase (20%) in cAMP concentration was achieved when 10^{-4} M IBMX was added. Lower concentrations of IBMX (10^{-5} and 10^{-6} M)

caused a slight (7%) increase in cAMP concentration. The dose-dependent effect of IBMX on cAMP was found to be the same when measured after 4 (Table 5, experiment I) or 6 days of treatment (data not shown).

As for the effect of CT on cAMP induction, a maximal increase in cAMP concentration (56%) was achieved upon the addition of 20 ng/ml CT to a medium contain-
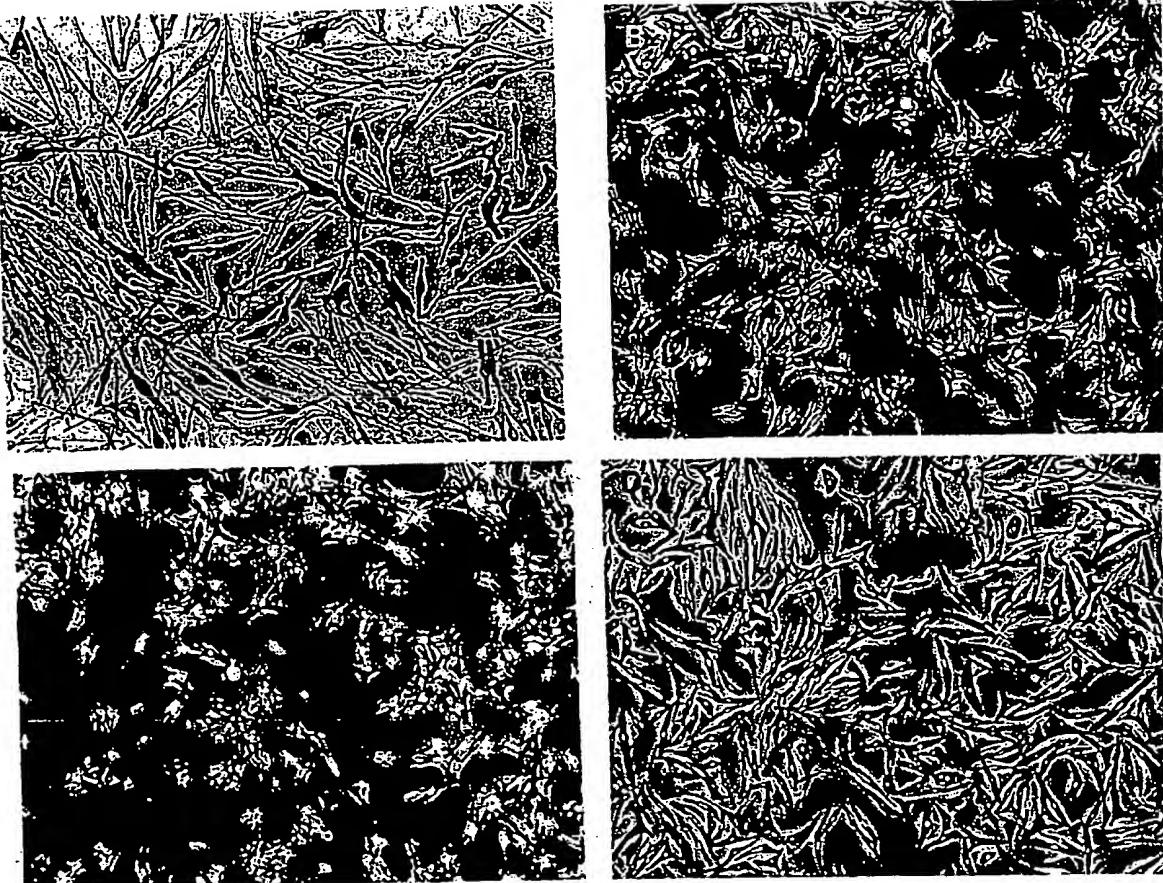
Fig. 5.   Light microscopic view (1,500X) of NHM grown in the presence of (A) 8 nM TPA, 20 ng/ml CT, and 10⁻⁴ M IBMX; (B) 1.2 ng/ml hrbFGF, 20 ng/ml CT, and 10⁻⁴ M IBMX; (C) 6 ng/ml hrbFGF, 20 ng/ml CT, and 10⁻⁴ M IBMX; and (D) 1.2 ng/ml hrbFGF without CT and IBMX.

ing 1.2 ng/ml hrbFGF and 10^{-4} M IBMX (Table 5, experiment II). The stimulatory effect of CT was measurable after 4 days of treatment (Table 5) and was greatly reduced after 6 days of treatment (data not shown).

DISCUSSION

Our results, and those previously reported by others, clearly show that agents that elevate cAMP concentration, such as CT and IBMX, greatly potentiate the mitogenic effect of TPA on NHM (Fig. 1; Eisinger and Marko, 1982; Halaban et al., 1986; Wilkins et al., 1985). In a series of dose–response experiments we found that TPA, known to be a necessary component of some media that support the growth of melanocytes, did not result in a remarkable proliferative rate in the absence of CT and IBMX (Fig. 1). In one study, however, Pittlekow and Shipley (1989) could not detect any enhancement of the proliferative response to TPA by CT or IBMX. One possible explanation for this is their use of pituitary extract in the culture medium, which may have resulted in a maximal mitogenic response. Whole bovine pituitary extract was found to be an essential

component for the growth of adult human melanocytes in the conditions described by Medrano and Nordlund (1990). We have found that the proliferation of NHM was maximally induced by 8 nM TPA, 200 ng/ml CT, and 10^{-4} M IBMX (Table 1, experiment II) and could not be further stimulated by increasing the concentration of TPA to 80 nM (data not shown).

Halaban et al. (1987, 1988) have shown that bFGF synthesized by human keratinocytes is mitogenic for NHM. We found that in the absence of CT and IBMX, NHM underwent one round of cell division in the presence of 0.6 ng/ml hrbFGF after 5 days of treatment (Fig. 2). Increasing the concentration of hrbFGF from 0.6 to 6 ng/ml result in a 30–40% increase in proliferation (Fig. 2). As noted with TPA, the concomitant presence of CT and IBMX greatly augmented the mitogenic response to hrbFGF at all the concentrations of hrbFGF tested (Fig. 2). We have found that in all the NHM strains tested (n = 7), NHM replicated every 24–36 hours in the presence of 1.2 ng/ml hrbFGF, 20 ng/ml CT, and 10^{-4} M IBMX. The positive contribution of CT and IBMX to the mitogenic effect of bFGF is in agree-

TABLE 6. Summary of the effects of TPA and hrbFGF a) individually, b) in the presence of either CT or IBMX, or c) in the concomitant presence of CT and IBMX[1]

	Cell proliferation	Tyrosinase activity	cAMP concentration
TPA (a)	+	+	0
+CT (b)	++	++	+
+IBMX (b)	++	++	+
+CT, +IBMX (c)	++++	+++	+++
hrbFGF (a)	+	−	±[2]
+CT (b)	−	−	ND
+IBMX (b)	−	+	ND
+CT, +IBMX (c)	+++	++++	++[3]

[1]hrbFGF, human recombinant basic fibroblast growth factor. ND, not done. For other abbreviations, see footnote 1 to Table 1.
[2]± Denotes a slight increase.
[3]This effect of CT could be detected after 4 days, and is reduced after 6 days of treatment.

centration of TPA than induction of the proliferative pathway. Our data shows that using 8 nM TPA in the presence of 20 ng/ml CT and 10^{-6} M IBMX results in a tyrosinase activity that cannot be further upregulated (Table 1, experiment I). This limits the usefulness of these melanocytes in studies aimed at investigating the regulation of pigmentation. For this reason, we sought to substitute TPA in the growth medium by hrbFGF, a factor that resulted in an optimal proliferative rate and at the same time allowed for further upregulation of tyrosinase activity.

While investigating the effect of hrbFGF on tyrosinase, we found that increasing the concentration of hrbFGF above 1.2 ng/ml to 3 and 6 ng/ml resulted in a dose-dependent decrease in the activity of this enzyme (Fig. 4). It is unlikely that the effect of hrbFGF on tyrosinase activity is due to a dilution of the enzyme due to rapid cellular proliferation, since this decrease was also observed in the absence of CT and IBMX, when NHM did not proliferate rapidly (Fig. 2). It is well known that one pathway by which tyrosinase activity is stimulated involves the activation of PKA by cAMP. It has been reported that bFGF can serve as a substrate for PKA (Feige and Baird, 1989). One explanation for the observed effect of hrbFGF on tyrosinase activity is that hrbFGF may act as a substrate for PKA and may thus competitively inhibit the phosphorylation of a yet unidentified protein that may be essential for the stimulation of tyrosinase.

Our observation that neither CT nor IBMX alone could contribute to the mitogenic effect of hrbFGF suggests that the additive effect of these agents on cAMP is essential for enhancing proliferation (Table 2). We have also observed that the presence of IBMX is required for increasing tyrosinase activity, and that in its absence, CT at 20 ng/ml does not have a stimulatory effect (Table 2, experiment I). This finding suggests that IBMX may elicit its stimulatory effect on tyrosinase by a mechanism that is cAMP independent (Table 2, experiment I).

We have observed dramatic morphologic changes induced by increasing the concentration of hrbFGF in the culture medium in the concomitant presence of CT and IBMX. Increasing the concentration of hrbFGF from 1.2 to 3 or 6 ng/ml resulted in increased dendricity (Fig. 5C). It has been shown that bFGF induces the

proliferation of endothelial cells and dendrite formation by PC 12 cells by binding to the extracellular matrix, in particular to heparin sulfate proteoglycan (Rogelj et al., 1989). The possibility exists that the mitogenic and morphologic effects elicited by bFGF on NHM are mediated by this mechanism. On the other hand, deprivation of NHM grown in the presence of hrbFGF of CT and IBMX caused them to become bipolar and to have shorter dendrites (Fig. 5D). It is known that agents that increase cAMP also increase the dendricity of normal and malignant melanocytes (Kitano, 1973; Hirobe, 1978; Marwan et al., 1985). Thus it is not surprising that the removal of CT and IBMX would have the opposite effect.

We have investigated the role of cAMP in mediating the effects of TPA, hrbFGF, CT, and IBMX on proliferation and tyrosinase activity. The interaction between TPA, CT, and IBMX in the expression of the proliferative and melanogenic capacity of NHM strongly suggests that proliferation and melanogenesis are regulated by two signal transduction mechanisms: the PKC and the cAMP/PKA pathways. TPA is known to bind to PKC, and cholera toxin and IBMX are known to increase intracellular cAMP by stimulating adenylate cyclase and inhibiting phosphodiesterase activity, respectively (Castagna et al., 1982; Enomoto and Gill, 1980; Russel and Pasean, 1974). We have observed that in the presence of CT and IBMX, TPA increased cAMP concentration in a dose-dependent fashion (Table 3, experiment I). However, in the absence of the former two agents, the stimulatory effect of TPA on cAMP was not evident (data not shown). Both CT and IBMX stimulated cAMP levels dose-dependently (Table 4). The TPA-induced increase in cAMP, in the presence of CT and IBMX, might represent another example of a positive interaction or "cross talk" between the PKC and the cAMP/PKA signal transduction pathways, as reported previously using other cellular systems (Yoshimasa et al., 1987). Our results indicate that TPA, CT, and IBMX interact positively to increase cAMP and to stimulate both proliferation and tyrosinase activity.

We have found that increasing hrbFGF from 1.2 to 6 ng/ml did not result in a remarkable increase in cAMP concentration (Table 3, experiment I). In the presence of hrbFGF, a significant increase in cAMP in response to IBMX was only evident at 10^{-4} M (Table 5, experiment I). We have also observed that stimulation of cAMP formation by CT was measurable after 4 days (Table 5, experiment II) and was greatly diminished after 6 days of treatment (data not shown). These results suggest that in the presence of hrbFGF, IBMX may not efficiently inhibit phosphodiesterase activity, thus resulting in rapid degradation of cAMP that is synthesized in response to CT.

In summary, our results show that the effects elicited by hrbFGF and its means of interaction with CT and IBMX differ from those of TPA (Table 6). While both hrbFGF and TPA are mitogenic to NHM, hrbFGF diminishes while TPA increases tyrosinase activity. In a medium supplemented with TPA, the addition of either CT or IBMX results in increased proliferation, tyrosinase activity, and cAMP concentration. However, in a medium containing hrbFGF, the concomitant presence of CT and IBMX is obligatory for the potentiation of

proliferation, and the presence of IBMX seems necessary for the stimulation of tyrosinase activity by CT. In the presence of hrbFGF and CT, IBMX does not cause a remarkable increase in cAMP level. Moreover, the dose-dependent stimulatory effect of CT on cAMP formation in the presence of hrbFGF and IBMX is evident after 4 but not after 6 days of treatment. Whether the increase in cAMP in NHM is transient, as reported for murine melanoma cells following treatment with α-melanocyte stimulating hormone (MSH), remains to be determined by measuring the effects of mitogens at different time points (Fuller and Viskochil, 1979). The differences listed above make it necessary to investigate the mechanism of action of hrbFGF on NHM. Basic FGF is known to activate a tyrosine kinase (Coughlin et al., 1988). Whether this is an alternative mechanism by which melanocyte proliferation and melanization are regulated is still unknown.

We postulate that cAMP plays a role in modulating both proliferation and melanogenesis, since reduction of cAMP level (e.g., by removal of CT and IBMX from the growth media) diminishes both. Cyclic AMP induction alone is not sufficient for melanocyte proliferation. Another mechanism by which NHM proliferation and/or melanogenesis may be regulated is the PKC/ phosphoinositol pathway. It has been reported that oleoyl-acetyl glycerol (OAG), a synthetic analog of diacylglycerol that activates PKC, results in increased melanogenesis, and that inhibition of PKC abrogates this effect (Gordon and Gilchrest, 1989). In our hands, OAG is also mitogenic to NHM and can replace TPA or hrbFGF in the NHM growth medium (unpublished data). Thus the role of PKC does not seem to be restricted to the regulation of melanogenesis. Whether activation of PKC or its downregulation by chronic exposure to TPA takes place in NHM remains to be determined. Also, the possible preferential expression of different PKC species and the status of PKA activation under different culture conditions needs to be examined.

ACKNOWLEDGMENTS

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Activation of melanogenesis by vacuolar type H$^+$-ATPase inhibitors in amelanotic, tyrosinase positive human and mouse melanoma cells

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Abstract In this study, we describe the activation of melanogenesis by selective vacuolar type H$^+$-ATPase inhibitors (bafilomycin A1 and concanamycin A) in amelanotic human and mouse melanoma cells which express tyrosinase but show no melanogenesis. Addition of the inhibitors activated tyrosinase within 4 h, and by 24 h the cells contained measurable amounts of melanin. These effects were not inhibited by cycloheximide (2 μg/ml) which is consistent with a post-translational mechanism of activation. Our findings suggest that melanosomal pH could be an important and dynamic factor in the control of melanogenesis in mammalian cells. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Melanogenesis; Tyrosinase activation; Melanosomal pH

1. Introduction

Melanin production by mammalian pigment cells is a process involving a series of complex cellular events. Despite the recent cloning and characterisation of several enzymes and structural proteins (tyrosinase, tyrosinase-related proteins type 1 and 2 (TRP-1 and -2), silver locus 100 kDa protein (Gp100), melanoma-associated antigen recognised by T cells (MART)) that are involved in the synthesis of melanin [1], our understanding of this process and its regulation is still far from clear. The copper containing metalloenzyme tyrosinase (EC 1.14.18.1) which is specific to melanocytes and catalyses the initial steps in the melanogenesis pathway is considered to be the rate limiting enzyme for melanin synthesis. Its absence, as occurs in the hypopigmentary disorder tyrosinase negative albinism, results in a complete absence of melanin.

As the key determinant for melanin synthesis, tyrosinase is under a sophisticated control by transcriptional and translational mechanisms. Recently, there has been interest in the role of post-translational events in the regulation of tyrosinase activity [2–5]. It is recognised that variations in tyrosinase activity in different pigment cells cannot be explained on the basis of enzyme abundance [6]. For instance, amelanotic and pigmented subclones of melanoma cells can have similar levels of tyrosinase protein [7] and the same has been reported for

melanocytes from light Caucasian and black skin [8]. Furthermore, it has been shown that the stimulation of tyrosinase activity and melanin synthesis occurs faster in cytoplasts than in nucleated cells [9]. These results suggest that in some pigment cells tyrosinase is present in a catalytically inactive state and that its activation could be an important control point for melanin synthesis.

Melanin production takes place within specialised intracellular organelles known as melanosomes. Since catalytic domains of tyrosinase are located on the inner side of the melanosomal membrane, its activity could be dependent upon the melanosomal environment. There is evidence that melanosomes are closely related to lysosomes. For instance, both organelles contain the same structural proteins (e.g. lysosome-associated membrane protein, acidic hydrolases, vacuolar type proton pumps) [10,11] and both are affected in genetic disorders such as the Chediak–Higashi and Hermansky–Pudlak syndromes [12,13]. More recently, it has been suggested that the melanosome is accessible to endosomal transport and represents a highly specialised lysosome rather than a completely unique structure [14,15]. This hypothesis is supported by studies which have shown that when human diploid fibroblasts are transfected with tyrosinase and TRP-1, the enzymes co-localise in lysosomes and melanogenesis occurs [16,17].

There are several reports that melanosomes are acidic organelles and when mature can have a pH as low as 4.0 [18,19]. It has been assumed that this low melanosomal pH facilitates melanogenesis [20–22]. However, this is not consistent with the finding that mammalian tyrosinase has optimal enzymatic activity at neutral pH [26–28] and shows little activity at pH < 5. On the contrary, there are reports that agents which raise lysosomal pH can increase tyrosinase activity. Thus increases in tyrosinase activity have been reported in pigmented mouse B16 melanoma cells in response to 10 mM NH$_4$Cl and the proton/metal ion exchanging ionophores, nigericin and monensin [26,27]. This stimulatory activity occurred in intact melanoma cells but not cell lysates, emphasising the importance of intact intracellular membranes. It was proposed that an elevation of melanosomal pH could be responsible for the increase in tyrosinase activity, although other possibilities were not excluded. Ammonium chloride has been shown to inhibit degradation of tyrosinase and increase its half-life by 3-fold [28], and ionophores could act by the facilitating exchange of univalent metal ions.

The purpose of this study was to investigate the effect of increasing melanosomal pH on melanogenesis. It has been shown that the selective vacuolar type H$^+$-ATPase inhibitors bafilomycin A1 (BafA1) and concanamycin A that target acidic intracellular compartment pH [29,30] are effective in increasing pH in these compartments in melanocytic cells

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Abbreviations: TRP-1 and TRP-2, tyrosinase-related proteins type 1 and 2; Gp100, silver locus 100 kDa protein; MART, melanoma-associated antigen recognised by T cells; BafA1, bafilomycin A1
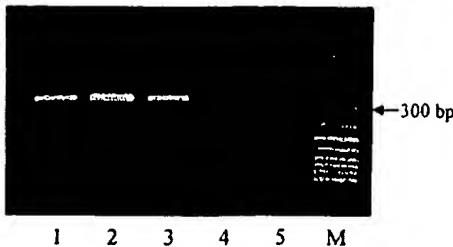
Fig. 1. RT-PCR for human and mouse tyrosinase mRNA. Human melanocytes (positive control), lane 1; B16-G4F, lane 2; FM3, lane 3; FM57, lane 4; FM81, lane 5; 100 bp 'ladder' MW marker, lane M.

(N. Smit, personal communication). In the present study, we examined the effects of BafA1 and concanamycin A in amelanotic but tyrosinase positive melanoma cells.

2. Materials and methods

2.1. Cells and cell cultures

Human melanoma cell lines FM3, FM57 and FM81 established from metastatic melanoma nodules were gifts from Dr A.F. Kirkin. The amelanotic mouse B16-G4F line was a gift from Prof. A. Eberle. Melanoma cells were cultured in RPMl 1640 medium supplemented with foetal bovine serum (10%). BafA1 and cycloheximide were obtained from Sigma, geneticin (G418) from Gibco BRL and concanamycin A from Calbiochem.

2.2. Reverse transcription (RT)-PCR

Total RNA was isolated using TRI Reagent (Sigma). The quality of RNA was tested by the A_{260}/A_{280} ratio and 1.5% agarose gel electrophoresis. cDNA synthesis was performed with MoMLV reverse transcription kit (Fermentas) following the manufacturer's instruction using approximately 1 µg of total RNA and 500 pmol of oligo(dT$_{16}$) primers in 20 µl of reaction mixture. 1 µl of cDNA was used for PCR amplification in 50 µl of reaction mixture containing: 1×PCR buffer (50 mM KCl, 20 mM Tris pH 8.4), 2.5 mM MgCl$_2$, 10 pmol of each primer, 200 µM dNTP (Fermentas) and 1.0 U of recombinant Taq polymerase (Fermentas). The parameters used for the 'touch down' amplification were initially 10 cycles of 94°C for 30 s, 65°C (decreasing 0.5°C per cycle) for 30 s and 72°C for 1 min followed by 25 cycles with the annealing temperature of 60°C. Primers specific for 284 bp fragment of human and mouse tyrosinase were used (forward: TTGGCAGATTGTCTGTAGCC; reverse: AGGCATTGTGCATG-CTGCTT). The following primers were used to test human melanoma lines only: 792 bp fragment of TRP-1 (forward: CACAAAGAG-CTGCAAACC, reverse: AGGAAGGGAGAAAGAAGG); 873 bp fragment of TRP-2 (forward: CCCTACATCCTACGAAACC, reverse: TTGAGAATCCAGAGTCCC); primers specific for Gp100 and MART were used as described [31].

2.3. Tyrosinase activity

Tyrosinase activity was assayed by measuring the production of radioactive water from tritiated L-tyrosine as described [26]. Briefly, L-[3,5-^{3}H]tyrosine (46 Ci/mmol, from Amersham) was diluted in cell culture medium to produce a final concentration of 5 µCi of L-[3,5-^{3}H]tyrosine per ml. Cells were returned to incubator and 200 µl aliquots of medium were collected after periods of 4, 8 and 24 h. Protein was precipitated by adding 200 µl of 15% (w/v) trichloroacetic acid, and the supernatant treated with 50 mg of Norit A charcoal slurry to remove labeled tyrosine.

2.4. Melanin and protein assays

The amount of melanin was assayed by dissolving a washed cell pellet directly in 1 ml of Soluene 350 (Packard Instruments) and incubating the samples for 2 h at 60°C. Synthetic melanin (Sigma) was used to construct a standard curve for the range of 1-50 µg of melanin per ml of Soluene 350. Melanin absorbance was measured at 475 nm wavelength. Protein concentrations were determined with an assay kit, based on the Lowry assay (Bio-Rad).

3. Results

3.1. Cell characterisation by RT-PCR

Both the B16-G4F and FM3 melanoma cells were found to express tyrosinase mRNA. Although the FM57 and FM81 melanoma cells expressed melanocyte markers Gp100, MART, TRP-1 and TRP-2, they showed no detectable expression of the tyrosinase gene (Fig. 1). The FM3 melanoma cells also expressed Gp100, MART and TRP-2 but no TRP-1.

3.2. Tyrosinase activity and melanin

The levels of tyrosinase activity in FM3 and B16-G4F cells were found to be almost undetectable under control conditions. The FM57 and FM81 melanoma cells that were included as controls contained no tyrosinase activity. No melanin was detected in any of melanoma lines under control conditions.

BafA1 and concanamycin A produced dose-related increases in tyrosinase activity in the tyrosinase positive cell lines FM3 and B16-G4F and as seen in Fig. 2, concanamycin A was the more potent of the two compounds. This order of potency correlates with their respective binding affinities to vacuolar type H$^+$-ATPase (Calbiochem general catalogue, 2000). Concentrations of both compounds in excess of 20 nM were cytotoxic (data not shown). Submaximal concentrations of 3 nM concanamycin A and 10 nM BafA1 were therefore used in all subsequent experiments and as shown in Fig. 2, these concentrations have similar potencies in stimulating tyrosinase activity. In response to 10 nM BafA1 and 3 nM concanamycin A, cell lines showed a rapid and statistically
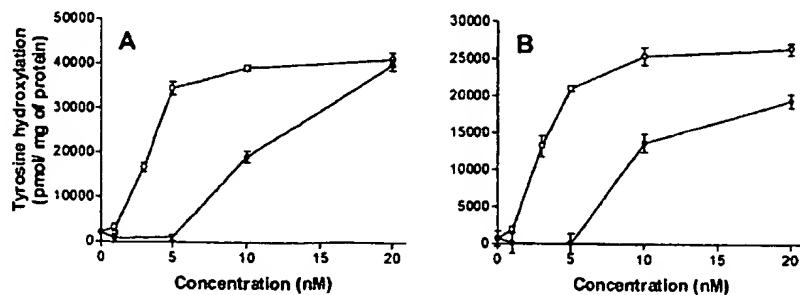
Fig. 2. Effect of vacuolar type H$^+$-ATPase inhibitors on tyrosinase activity in FM3 (A) and B16-G4F (B) cells. The cells were incubated for 24 h with varying concentrations of concanamycin A (O) and BafA1 (●). Doses higher than 20 nM were toxic for cells.
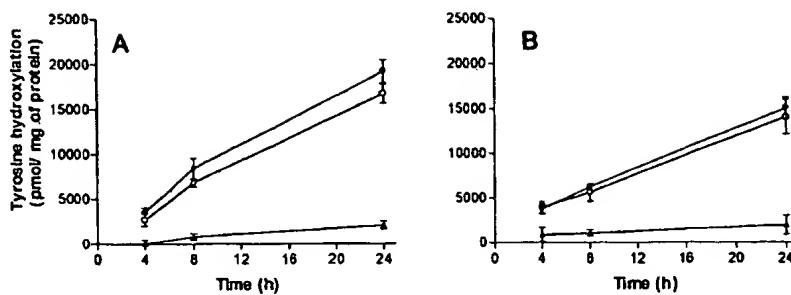
Fig. 3. Time course of tyrosinase activation by vacuolar type H⁺-ATPase inhibitors in FM3 (A) and B16-G4F cells (B). The cells were incubated for the indicated periods with: (▲) complete medium (control); (O) 3 nM concanamycin A; (●) 10 nM BafA1.

significant increase in tyrosinase activity which lasted for the 24 h duration of the experiment (Fig. 3). At this time, the cell pellets were visibly darker and contained measurable amounts of melanin (Fig. 4). The protein synthesis inhibitor cyclohex-imide (2 µg/ml) had no effect on these increases in tyrosinase activity (Fig. 5) and melanin (data not shown). Incubation of the cells with a high dose of cycloheximide (10 µg/ml) alone or the cell cycle arresting agent geneticin (200 µg/ml) failed to induce tyrosinase activity and melanin production (data not shown).

Incubation of the tyrosinase negative FM57 and FM81 melanoma cells with the same concentrations of BafA1 and concanamycin A produced no detectable amounts of [³H]water or melanin.

4. Discussion

A number of studies have suggested that tyrosinase in pigment cells does not necessarily exist in a catalytically optimal state [6,32]. There have also been several reports that activation of this enzyme can occur independently of increases in its synthesis [5,33]. In the present study, the amelanotic, but tyrosinase positive, FM3 and B16-G4F melanoma cells were found to contain almost undetectable levels of tyrosinase activity and intracellular melanin was absent. However, in response to BafA1 and concanamycin A, there was a rapid increase in tyrosinase activity followed by melanin production. The levels of melanin production and tyrosinase activity

in the FM3 and B16-G4F cells in response to BafA1 and concanamycin A were comparable to those that we observe in pigmented human melanocytes and B16-F1 mouse melanoma cells, respectively. A high dose of cycloheximide and a cell cycle arresting concentration of geneticin had no effect on melanogenesis, eliminating the possibility that the increases in melanogenesis were associated with non-specific cytostatic effects of BafA1 and concanamycin A. The fact that the melanogenesis response was not abolished by cycloheximide makes it unlikely that tyrosinase abundance was increased and points towards an activation of pre-existing enzyme. A non-genomic mechanism is therefore implicated and this is consistent with the rapid kinetics of tyrosinase activation. Thus our findings support the view that under normal conditions FM3 and B16-G4F melanoma cells contain tyrosinase but because this is inactive, melanin synthesis is suppressed and the cells exhibit an amelanotic phenotype. However, in response to the vacuolar type proton pump inhibitors which induce an increase in the pH of acidic intracellular compartments, tyrosinase is activated and melanin production initiated.

It has previously been reported that tyrosinase activity in vitro is minimal at pH < 5 and that the enzyme is irreversibly inactivated at a pH lower than 4 [23–25]. However, as the pH rises, tyrosinase activity increases, reaching a maximal level at near pH 7.0. This range of proton concentration (pH 5.0–7.0) corresponds well with the variations of pH inside organelles of the endosomal/lysosomal pathway that are created and maintained by proton pumps. As melanosomes are closely related
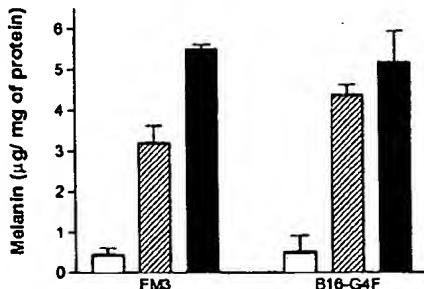
Fig. 4. Melanin production in response to vacuolar type H⁺-ATPase inhibitors. Cells were collected after 24 h incubation in complete medium (open bar) containing 3 nM concanamycin A (striped bar) or 10 nM BafA1 (closed bar). The control values are below the level of assay sensitivity (< 0.5 µg/mg of protein).
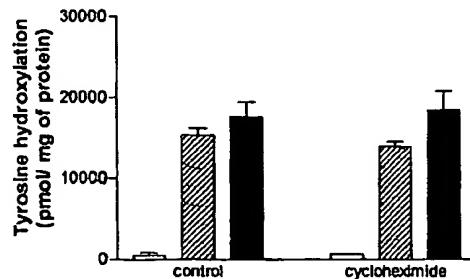
Fig. 5. Effect of cycloheximide on tyrosinase activation by vacuolar type H⁺-ATPase inhibitors in FM3 cells. Cells were incubated for 24 h in complete medium (open bar) containing 3 nM concanamycin A (striped bar) or 10 nM BafA1 (closed bar) with or without cycloheximide (2 µg/ml).

to this group of acidic organelles, it is possible that they show similar variations of pH and this could have physiological importance for melanogenesis through a non-genomic control of tyrosinase activity. It is of interest that cyclic AMP, which has been reported to increase lysosomal pH [34], is a well-recognised stimulator of melanogenesis. Melanogenesis also occurs in response to ultraviolet (UV) irradiation and increases have been observed in melanoma cells as early as 1–3 h after UV irradiation [35]. The speed of this response would suggest that non-genomic mechanism operates before de novo synthesis of tyrosinase occurs. Whether this rapid increase in melanin synthesis in response to UV is induced by a change in melanosomal pH remains to be investigated. Nevertheless, our results would suggest that melanosomal pH could function as a 'control switch' providing pigment cells with a fast and dynamic regulatory mechanism for melanogenesis.

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Pharmaceutical Dosage Forms and Drug Delivery Systems

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INDUCTION OF PIGMENTATION IN MOUSE FIBROBLASTS
BY EXPRESSION OF HUMAN TYROSINASE cDNA

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Cells of the melanocyte lineage are distinguished by their capacity to synthesize the pigment melanin. Production of melanin is primarily regulated by the enzyme tyrosinase (monophenol, 3,4-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), and it is presumed that melanin synthesis can be regulated at a number of levels that control both the amount of melanin synthesized and the type of melanin produced. Melanin synthesis occurs principally in specialized organelles, the melanosomes. Thus, the synthesis of melanin is usually restricted to melanocytic cells that contain melanosomes.

In this report, we describe isolation of a full-length cDNA clone encoding human tyrosinase by using a probe homologous to the Pmel 34 cDNA sequence described by Kwon et al. (1). We have transfected and expressed this new human tyrosinase cDNA clone in mouse fibroblasts, and have induced pigmentation in a cell type that does not normally synthesize melanin. Levels of tyrosinase activity in transfected fibroblasts were equivalent to tyrosinase levels in highly pigmented human melanoma cell lines. These tyrosinase-positive fibroblast cell lines demonstrate that melanin synthesis can take place in cells that do not have melanosomes and, therefore, provide a tool for studying the regulation, transport, and processing of tyrosinase.

Materials and Methods

Cell Culture and Cell Lines.   Melanoma cell lines were established as previously described (2). TK⁻ L929 cells (mouse fibroblasts) (3) were used for transfection experiments. Cell lines were maintained in Eagle's MEM supplemented with 2 mM glutamine, 0.1 mM nonessential amino acids (aa)', 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS (complete medium). Cells were passaged with trypsin (1 mg/ml) and EDTA (0.2 mg/ml). All cultures were checked regularly for the presence of mycoplasma and contaminated cultures were discarded.

EM.   Cell pellets were fixed in Karnofsky's fixative overnight, rinsed in PBS for 1 h, and then post-fixed for 1 h in 1% osmium tetroxide-PBS solution. Cell pellets were dehydrated in graded ethyl alcohol followed by propylene oxide, and embedded in Maraglas-D.E.R. 732 epoxy resin (Dow Corning Corp., Midland, MI). For orientation, 1-μm thick sections were

stained with borate-buffered 1% toluidine blue. Thin sections were stained with uranyl acetate followed by lead citrate and were examined with an electron microscope (410 LS; Phillips Electronic Instruments, Inc., Mahwah, NJ).

cDNA Library and Screening.   A cDNA library was constructed from 3 μg of poly(A)⁺ selected mRNA (4) prepared from the human melanotic melanoma cell line SK-MEL-19 (2). Full-length cDNA was synthesized, rendered blunt ended using Klenow enyzme, and tailed with Eco RI linkers (New England Biolabs, Inc., Beverly, MA) (5). The cDNA was then size fractionated on Ultrogel Aca 34 (Pharmacia Fine Chemicals, Piscataway, NJ) (6). cDNA molecules >800 bp were used to construct a library of 3 × 10⁵ recombinants in the λ phage vector gt10 (7). For screening, a 50-base oligonucleotide probe (50-mer, shown below) based on the 5′ terminal coding region of the human tyrosinase Pmel 34 cDNA clone (1) was used: 5′ GTTCTTAGAGGAGACACAGGCTCTAGGGAAAATGGCCAGCGGAGGTCTGGA 3′.

The oligonucleotide was synthesized on a DNA synthesizer (310 A; Applied Biosystems, Inc., Foster City, CA). The probe was end labeled with γ-[³²P]ATP and T4 polynucleotide kinase (4). Prehybridization and hybridization were carried out at 48°C for 4 and 18 h, respectively, in 6× NaCl/EDTA/Tris (NET) (1× NET is 0.15 M NaCl, 1 mM EDTA, and 15 mM Tris-HCl, pH 8), 0.1% SDS, and 5× Denhardt's solution (0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), and 100 μg/ml of denatured salmon sperm DNA. Duplicate filters were washed in 6× NET, 0.1% SDS at room temperature, followed by stringent washes at 55°C and 60°C. The filters were then autoradiographed for 4 h at −70°C.

DNA Sequencing.   Plaque-purified phage DNA was restricted with Eco RI, and cDNA inserts were subcloned into the plasmid vector pUC 18 (8). Recombinant plasmids and deletion subclones subsequently obtained by digestion with exonuclease III/Mung Bean nuclease (9) were sequenced by the dideoxynucleotide chain termination method (10).

Northern Blot Analysis.   Poly (A)⁺-mRNA (4 μg) was fractionated on 1% formaldehyde denaturing agarose gels (4), transferred to Gene Screen Plus membranes (New England Nuclear, Boston, MA), and hybridized to a ³²P-labeled cDNA probe. The filters were washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and 1% SDS, then stringent washes were carried out at 55°C in 1× SSC, 1% SDS, and at 65°C in 0.1× SSC, 1% SDS, for 15 min each.

Transfection Experiments.   The cDNA inserts were subcloned into the Eco RI site of the expression vector pcEXV-3, which allows expression of cDNA under the control of SV40 early region promoter and enhancer sequences (11). Expression plasmids containing cDNA inserts in opposite orientations (5′→3′ or 3′→5′) were constructed. Sense and antisense oriented plasmids were designated pcTYR and pcTYW, respectively. L929 cells were cotransfected by the calcium phosphate precipitation technique (12) with pUC 18, pcTYW or pcTYR, the pSV2 *neo* plasmid, and high molecular weight carrier DNA from L929 cells. Selection of transfectants was started on day 3 after transfection with 1 mg/ml of the antibiotic G418 (Sigma Chemical Co., St. Louis, MO). Complete medium with G418 was replaced every 3 d, and colonies appearing on days 10–14 were isolated using cloning rings and were then expanded. The mouse origin of transfected cell lines was confirmed by positive anti-mouse Ig mixed hemadsorption assays using H100-5R28, an mAb directed against H-2K^k (mouse MHC class I antigens) (13), and lack of reactivity with mAb M3-68 (14) or AJ2 (15), which recognize virtually all human melanoma cells but not L929 cells (data not shown).

Serological Reagents and Assays.   CF21 (IgG1) and TA99 (IgG2a) are mAbs, which have been previously described (16), that recognize distinct antigens in human melanosomes. The mAb 2G10 (IgG2a) (17) was a generous gift from Dr. P. G. Natali, (Regina Elena Cancer Instituto, Rome, Italy). This antibody recognizes a 75-kD intracellular glycoprotein of pigmented melanotic cells (17). mAb AJ2 (IgG1) recognizes the β subunit of human integrin molecules (15, 18). Rabbit antityrosinase antiserum was raised by immunization with purified mouse tyrosinase (19). Briefly, tyrosinase was purified by DEAE ion exchange chromatography followed by sequential discontinuous PAGE. The anti-mouse Ig hemadsorption assays and the indirect immunofluorescence studies were performed as described (2, 20).

Immunoprecipitations.   Cells were labeled with ³⁵S-methionine (ICN Radiochemicals, Irvine, CA) for 16 h in methionine-free complete medium containing 2% dialyzed FCS, and lysed in 50 mM Tris, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF. The lysates were precleared

twice by incubation with 5 μg/ml of protein A-Sepharose (Pharmacia Fine Chemicals) for 30 min at 4°C. Immunoprecipitations were performed by incubating the lysates with antibodies, followed by addition of protein A-Sepharose. The immunoprecipitates were extensively washed and analyzed for molecular size by SDS/PAGE (21) under reducing conditions.

Tyrosinase Activity and Melanin Assays. Cells were solubilized in PBS, 1% NP-40, pH 6.8, and centrifuged to obtain clear supernatants. Tyrosine hydroxylase activity was assayed using a modification of the method described by Pomerantz (22). Briefly, the reaction mixture contained 1 μCi/ml [³H]tyrosine (54.2 Ci/mMol) in PBS, 1% NP-40, 0.1 mM L-tyrosine, and 0.1 mM L-dihydroxyphenylalanine (L-DOPA). The reaction was carried out at 37°C for 1 h, and terminated by addition of 0.2 ml of a charcoal suspension (100 mg/ml in 0.1 M citric acid). After 30 min on ice, the samples were centrifuged and an aliquot was counted in a scintillation counter (LS 9000; Beckman Instruments, Inc., Fullerton, CA). All assays were performed in duplicate. Controls included ³H₂O release measured in lysates from the human renal carcinoma cell line SK-RC-7 and reaction mixture in PBS, 1% NP-40 alone. Specific tyrosinase activity was calculated as follows: (³H₂O release by test cell lysate) − (³H₂O release by control reaction mixture in PBS). Protein concentrations were determined by the Bradford's dye binding method using the Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). For melanin assays, 3 × 10⁶ cells were solubilized in 0.5 ml Protosol (New England Nuclear) and kept on ice for 2 h. An absorption baseline was established using Protosol, and absorption spectra for cell extracts were determined between 320 and 450 nm and compared against a melanin control, 100 μg/ml, in Protosol.

Results

Isolation and Sequencing of the cDNA Clone BBTY-1. cDNA clones were isolated from a λ gt10 library derived from the pigmented human melanoma cell line SK-MEL-19 (see Materials and Methods). 10⁵ recombinant cDNA clones were screened and four reactive clones were plaque purified. The four cDNA inserts were subcloned into the plasmid vector pUC 18 and clones were designated pBBTY-1, -2, -3, and -4. Two clones, pBBTY-1 and pBBTY-2, each containing cDNA inserts of 2 kb, had restriction maps identical to each other and to that of Pmel 34, reported by Kwon et al. (1) (digested with Bgl II, Hpa II, Msp I, Nco I, Pvu II, and Taq I). The cDNA inserts in clones pBBTY-3 and pBBTY-4 were 1.7 and 1.8 kb, respectively. The restriction map of pBBTY-3 was different from those of pBBTY-1 and pBBTY-2 downstream of position 960 (a Pvu II restriction site). pBBTY-1 was subsequently sequenced and used for further experiments.

The nucleotide sequence of BBTY-1 (Fig. 1) contained a single open reading frame of 1,593 residues capable of encoding a 531-aa polypeptide with a derived molecular mass of 60.37 kD. A leader peptide of 19 aa was assigned to positions -19 through -1 (23). The processed core protein was predicted to have a molecular mass of 58.11 kD. Seven potential N-glycosylation signals (Asn-X-Ser/Thr) were predicted at positions 69, 94, 144, 213, 273, 320, and 354. Based on a hydrophobicity plot, according to the method of Kyte and Doolittle (24), a transmembrane region was predicted within a highly hydrophobic domain between aa positions 470 and 490. There was a 318 base 3' noncoding region that contained an atypical polyadenylation signal, AATTAAA (25). The nucleotide and aa sequences of BBTY-1 were nearly identical to the sequence of the Pmel 34 cDNA. (1). However, BBTY-1 contained an additional upstream 5' sequence, including a potential initiation codon not present in Pmel 34 (bases 1–7). There were also differences in the predicted aa sequence of BBTY-1 at positions 25–28, 162, 291, 356–361, 385, 478, and 503–512. The predicted molecular size of the processed protein coded by BBTY-1 was smaller than the processed

FIGURE 1. Nucleotide and predicted amino acid sequence of BBTY-1 cDNA. The nucleotide sequence is numbered in the 5' to 3' direction. Residues of a predicted signal peptide are indicated by negative numbers, and a cleavage site by a vertical arrow. Termination site (TAA) and polyadenylation signal (1869-1875) are underlined. Potential glycosylation sites are designated by dashed lines. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00819.

protein predicted from Pmel 34 (62.16 kD). Based on this sequence analysis, BBTY-1 was a candidate for a full-length cDNA clone encompassing a complete coding region.

Transcription of BBTY-1 in Human Melanoma Cells. BBTY-1 cDNA was used to detect mRNA transcripts in Northern blot analysis of a panel of melanoma cell lines, including those known to express tyrosinase activity as well as tyrosinase-negative melanomas. The major transcript detected was 2.4 kb, but a weaker signal was seen at 4.7 kb (Fig. 2). Three groups of melanomas were observed based on Northern blot analysis using poly (A)$^+$-selected RNA (data not shown). (*a*) mRNA was detected in nine pigmented melanomas that express tyrosinase activity; (*b*) no mRNA was detected in five nonpigmented, tyrosinase-negative melanomas; and (*c*) mRNA was detected in three nonpigmented, tyrosinase-negative melanomas. There was little or no difference in the intensity of mRNA signal detected in group *c* vs. *a*. No transcript was detected in mRNA from the B cell lymphoma cell line Daudi or from the T cell leukemia cell line HUT-78.

Melanin Synthesis in L929 Cells Transfected with BBTY-1. BBTY-1 was transfected into L929 mouse fibroblasts using the expression vector pcEXV-3 (11). L929 cells transfected with pcTYR (sense orientation) were designated LpcTYR. Control cells transfected with pcTYW (antisense orientation) were designated LpcTYW, and, with the plasmid pUC 18, were designated LpC. LpcTYR cells contained pigment, while no pigmentation was detected in LpcTYW, LpC, or untransfected L929 cells. As shown in Fig. 3, the cell pellets of LpcTYR clones were dark brown in contrast to the nonpigmented pellets of LpcTYW and LpC cultures.

Cell pellets of LpcTYR were more deeply pigmented when cultures were harvested at confluency. LpcTYR clones have continued to produce pigment for >5 mo in continuous culture. To confirm that the pigment in LpcTYR has the characteristics of melanin, absorption spectra of cell extracts from LpcTYR and control L929 cells were compared with those of extracts of the pigmented melanoma cell line SK-MEL-19 and purified melanin. LpcTYR and SK-MEL-19 extracts and melanin had identical patterns of absorption, with broad absorption from 360 to >450 nm; this absorption pattern was not observed with L929 cell extracts (data not shown). The absorption patterns by extracts of LpcTYR and SK-MEL-19 and melanin standard were identical to the previously described absorption spectra for melanin (26).
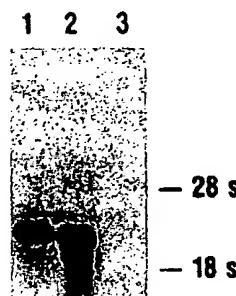
FIGURE 2. Northern blot analysis of poly (A)$^+$-selected RNA (4 μg/lane) from two pigmented melanoma cell lines that express tyrosinase and the B cell lymphoma cell line Daudi. The blot was hybridized with the ^{32}P-labeled insert of pBBTY-1. Lanes *1*, SK-MEL-23 melanoma; lane *2*, SK-MEL-19 melanoma; lane *3*, Daudi.

1   **2**   **3**   **4**

Figure 3.   Cell pellets of L929 cells transfected with sense BBTY-1 (LpcTYR cell line), antisense BBTY-1 (LpcTYW cell line), or pUC 18 plasmid (LpC cell line). LpcTYR and LpcTYW cell lines were transfected with BBTY-1-inserted sense or antisense into the expression vector pcEXV3. LpcTYR-1 and LpcTYR-2 are subclones of LpcTYR. (*1*) LpC cell pellet (nonpigmented); (*2*) LpcTYW (nonpigmented); (*3*) LpcTYR-1 (pigmented); and (*4*) LpcTYR-2 (pigmented).

Small clusters of cell containing dark cytoplasmic inclusions were observed throughout the LpcTYR culture by light microscopy (Fig. 4). These clusters of cells always comprised a minority of the culture population. Occasional black round cells were detected floating in the tissue culture medium, perhaps related to cytostatic or cytotoxic effects of melanin by-products, and the prevalence of these cells increased as the culture reached confluency. Transmission EM revealed that LpcTYR cells, but not control LpC cells, had cytoplasmic membrane-bound vesicles (Fig. 5) containing electron-dense material consistent with melanin. There was no evidence of melanosomal structural elements within LpcTYR cells or LpC cells.

Tyrosinase Activity in L929 Cells Expressing BBTY-1.   To confirm that the BBTY-1 product was human tyrosinase, tyrosine hydroxylase activity was measured in protein extracts of subclones of LpcTYR, LpcTYW, and LpC. Cell extracts from two subclones of LpcTYR, designated LpcTYR-1 and LpcTYR-2, expressed levels of tyrosinase activity that were comparable with levels in the pigmented human melanoma cell line SK-MEL-19 (Fig. 6). In contrast, extracts of LpcTYW and LpC contained no detectable tyrosinase activity.

Analysis of Expression of Melanosomal Antigens in LpcTYR Cells.   LpcTYR-2, SK-MEL-19 melanoma cells, and control L929 cells were metabolically labeled with ^{35}S-methionine and cell extracts were immunoprecipitated with rabbit antityrosinase antiserum or mAb TA99 (which detects the melanosomal antigen gp75). In addition,
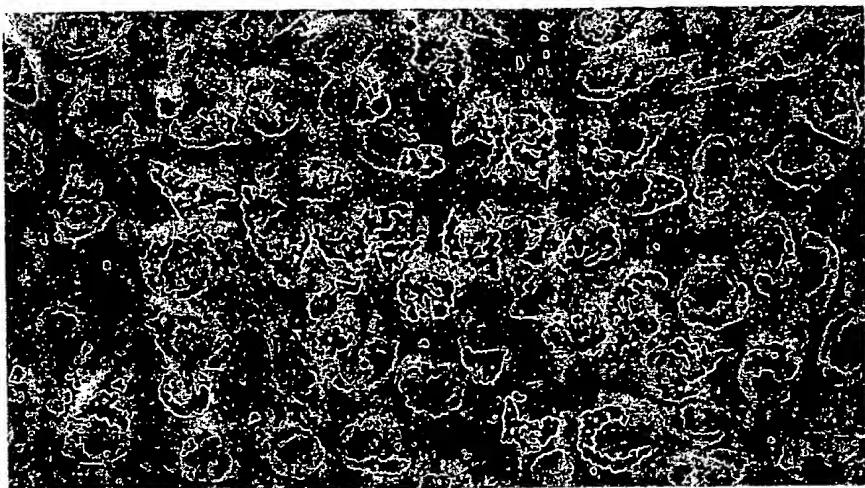
FIGURE 4. LpcTYR cells in culture. A nest of cells in the middle of the field contains large, pigmented cytoplasmic granules. (×320).

mAb 2G10 (from Dr. Pier Natali, Regina Elena Instituto, Rome), which is also directed against an intracellular 75-kD antigen expressed by pigmented melanoma cells (17), was tested. Antityrosinase antiserum detected a 75-kD protein in LpcTYR-2 cells and a protein of the same size in SK-MEL-19 melanoma cells (Fig. 7). The molecular size

A   B
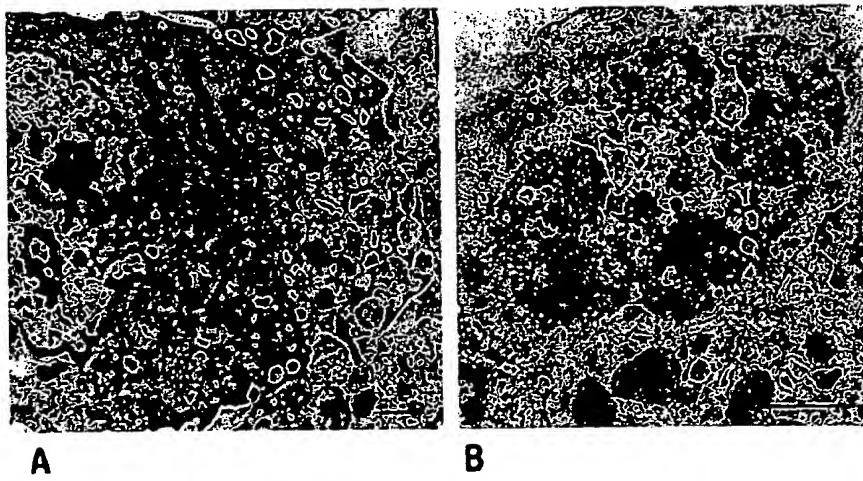
FIGURE 5. Transmission electron micrographs of segments of LpcTYR cells. (A) Cytoplasmic membrane-bound vesicles containing electron-dense material are indicated by arrows. One scale bar represents 1 μm. (×8,400). (B) Higher magnification field of a cytoplasmic membrane-bound vesicle containing pigment. One scale bar represents 1 μm. (×16,800).
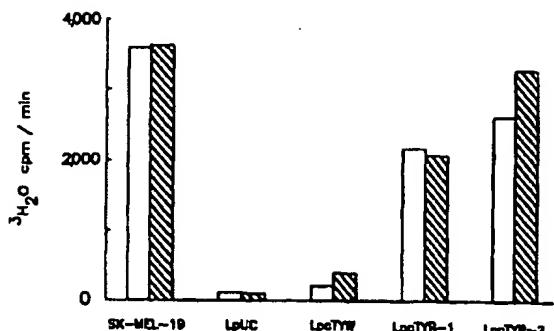
Figure 6. Expression of tyrosinase activity in cell extracts from: SK-MEL 19 melanoma; Lpc cells (transfected with pUC 18 plasmid); LpcTYR-1 cells (transfected with a BBTY-1 sense construct); LpcTYR-2 cells (transfected with a BBTY-1 sense construct); and LpcTYW cells (transfected with a BBTY-1 antisense construct). Tyrosine hydroxylase activity is expressed as cpm ^3H_2O/min/mg protein (□) or cpm ^3H_2O/min/5 × 10^6 cells (▨).
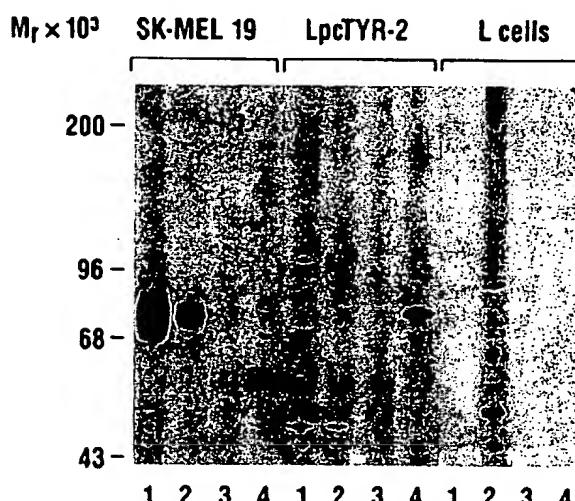
Figure 7. Immunoprecipitation of lysates from ^{35}S-methionine metabolically labeled SK-MEL-19 melanoma cells, LpcTYR-2 cells expressing BBTY-1, and L929 cells. Lane 1, mAb TA99; lane 2, mAb 2G10; lane 3, control rabbit sera; and lane 4, rabbit antityrosinase antisera. A 75-kD band is detected in SK-MEL-19 (with TA99, 2G10, and antityrosinase) and LpcTYR-2 cells (with antityrosinase). Molecular weight standards: Myosin M chain (200 kD); phosphorylase (96 kD); BSA (68 kD); and OVA (43 kD).

of tyrosinase in LpcTYR-2 and SK-MEL-19 cells corresponded to the size of glycosylated tyrosinase. A very faint band at ~75 kD was inconsistently detected in L929 cells with antityrosinase antiserum; this likely represents a crossreaction of polyclonal sera to a nontyrosinase molecule in L929 cells, since no tyrosinase activity or tyrosinase transcript was detected in these cells and cold lysates from L929 cells did not block immunoprecipitation of tyrosinase from LpcTYR-2 (data not shown).

No specific bands were detected by either mAb TA99 or mAb 2G10 in LpcTYR-2 extracts, although both antibodies precipitated a broad 75-kD band from melanoma SK-MEL-19 lysates. These results were confirmed using immunofluorescence assays. Neither mAb TA99 nor 2G10 stained LpcTYR cells but both reacted with SK-MEL-19 cells (Fig. 8). In addition, mAb CF21, directed against a melanosomal antigen of unknown molecular size, did not react with LpcTYR but stained SK-MEL-19 (Fig. 8). We conclude that mAbs TA99, CF21, and 2G10 identify antigens distinct from tyrosinase encoded by the BBTY-1 cDNA clone.
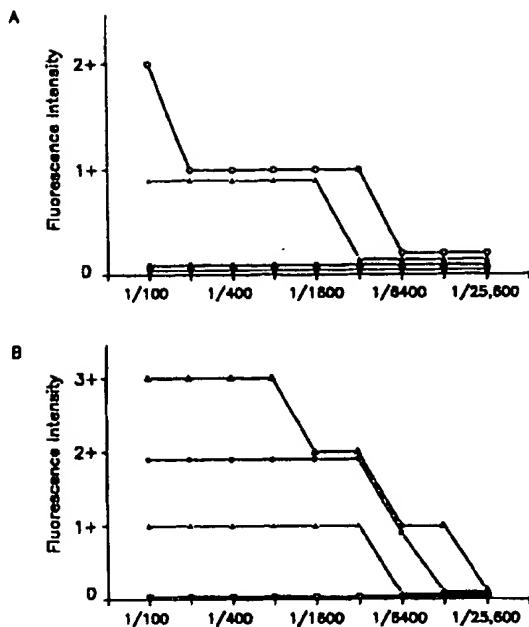
FIGURE 8. Indirect immunofluorescence assays for antigen expression by: (A) LpcTYR-2 cells expressing BBTY-1; and (B) SK-MEL-19 melanoma cells. mAb TA99 (anti-gp75) (Δ); mAb CF21 (antimelanosomal antigen) ($\bullet$); mAb H100-5R28 (anti-H-2^k) (O); and mAb AJ2 (antiintegrin; positive control) ($\blacktriangle$).

Discussion

Tyrosinase catalyzes the o hydroxylation of monophenols and oxidation of o-diphenols to o-quinones. In melanocytic cells, tyrosinase enzymatically converts tyrosine to DOPA, and DOPA to dopaquinone, leading to the spontaneous formation of the complex mixture of pigments known as melanin (27). The later steps in this pathway are not well characterized, and it has been suggested that a number of other factors, both catalytic and inhibitory, may regulate melanin synthesis and influence the species of melanin formed (28, 29). The complexity of pigment expression has been further highlighted by genetic studies in the mouse where >50 loci have been found to influence coat color (30). Thus, it is possible that a number of gene products, most not yet identified, can play a role in melanogenesis.

It is remarkable that transfected L929 fibroblasts not only stably expressed tyrosinase activity but were able to produce and package melanin. Melanin precursors are cytotoxic, and it has been presumed that melanocytic cells contain mechanisms, perhaps located within melanosomes, that protect from the effects of toxic intermediates. We suspect that melanin precursors were in fact cytotoxic in transfected L929 cells, and that cells producing substantial amounts of pigment were destined to die, based on the following observations: (a) only a subpopulation of transfected cells contained pigmented vesicles; (b) deeply pigmented, nonviable cells were observed floating in the supernatant of transfectant cultures; and (c) when transfected cells were cryopreserved and then thawed, pigmented cells were not initially detected but eventually repopulated the culture.

We have not yet analyzed in detail the synthesis or processing of human tyrosinase in transfected L929 cells. From our preliminary studies, it appears that human tyrosinase is glycosylated to a form that is identical in size to fully processed tyrosinase expressed in human melanocytic cells. It is likely that human tyrosinase was processed through the Golgi apparatus in L929 cells and transported to or remained in vesicles arising from the *trans*-Golgi. The nature and destination of these vesicles is not known. It is interesting to speculate that these vesicles might be precursors of melanosomes but that formation of melanosomes would depend on the products of other specialized genes.

The expression and regulation of tyrosinase has been the subject of extensive studies, but the formal identification of the gene that codes for tyrosinase has not been straightforward (reviewed in reference 31). Two distinct, and only distantly related, genes have been proposed as candidates for mouse tyrosinase, based on detection of mRNA of these genes in melanocytic cells and reactivity of the protein product with antibodies against tyrosinase (32, 33). Neither gene, however, was demonstrated directly to code for a product with tyrosinase activity. It is likely that antibodies used to detect the products of putative tyrosinase cDNA clones reacted with other molecules that copurified with tyrosinase. This situation was recently clarified by the identification of the mouse tyrosinase gene by Müller et al. (34) who isolated a cDNA clone, pmctyrl, that coded for transient expression of tyrosinase activity in transfection assays. No pigment synthesis was reported in transfected cells, possibly because assays were performed only shortly after transfection, because the recipient cells were different (an amelanotic melanoma and a breast carcinoma cell line), or because levels of tyrosinase activity appeared to be much lower than in mouse fibroblasts transfected with BBTY-1.

The candidate for the human tyrosinase gene, designated Pmel 34, has been reported by Kwon et al. (1). Kwon et al. (35) also recently described a mouse cDNA, MTY811C, isolated using Pmel 34. The gene product encoded by MTY811C was predicted to be 81% homologous to the protein encoded by Pmel 34. Both the human Pmel 34 and the mouse MTY811C correspond to the human counterpart of the mouse pmctyrl gene, and in fact, the pmctyrl clone was also isolated by screening a cDNA library from mouse melanoma cells with the Pmel 34 cDNA. The Pmel 34 cDNA clone was detected by screening a cDNA library with polyclonal antisera raised against hamster tyrosinase. Pmel 34 has been mapped to the c (albino) locus in the mouse, the presumed site of the tyrosinase structural gene or a gene that regulates tyrosinase expression. The nucleotide and predicted aa sequences of BBTY-1 and Pmel 34 are nearly identical. BBTY-1 contains an initiation codon that is not present in Pmel 34, and there are minor differences in nucleotide and predicted aa sequences. It is possible that some of these differences represent genetic polymorphism or somatic mutations (related to the source of cell types used to isolate cDNA, i.e., melanoma cells for BBTY-1 vs. melanocytes for Pmel 34). It is interesting to note that where there are distinct differences in sequences between BBTY-1 and Pmel 34, the sequence of BBTY-1 is very close or identical to the mouse pmctyrl tyrosinase sequence (e.g., aa 356–361 and 385).

Multiple transcripts of the tyrosinase gene have been found in mouse melanoma cells (36). The remaining transcripts are generated by alternative splicing leading to deletion of internal sequences, presumably by exon skipping or by selection of

internal splice sites. When these alternative transcripts have been expressed, they have not been found to encode active tyrosinase (34, 36). The BBTY-1 cDNA represents the human counterpart of the mouse pmctyrl transcript. Another cDNA clone that we isolated, BBTY-3, differs from BBTY-1 in its 3′ restriction map, possibly corresponding to an alternative transcript of the human tyrosinase gene.

We have asked what is the relationship of tyrosinase to the melanosomal/cytoplasmic antigens recognized by mAbs 2G10, TA99, and CF21. It has been shown that mAb 2G10 immunodepletes tyrosinase activity (37) and, therefore, possibly recognizes a molecule with tyrosinase activity. However, mAb 2G10 did not react with human tyrosinase encoded by BBTY-1, suggesting that mAb 2G10 recognizes a distinct molecule from the gene product of BBTY-1. TA99 mAb recognizes an acidic 75-kD glycoprotein (38), and the antigen recognized by TA99 is a candidate for tyrosinase, based on its expression in melanosomes, its molecular size, and charge. The finding that mAbs TA99 and CF21 did not react with L929 transfectants provides evidence that they do not recognize determinants coded for by the BBTY-1 human tyrosinase molecule. Further data suggest that mAb TA99 does not recognize tyrosinase: (a) mAb TA99 does not precipitate tyrosinase activity from melanoma cell extracts (39, 40); (b) the TA99 antigen, gp75, is generally coexpressed with tyrosinase activity, but there are examples of gp75⁺ melanoma cell lines that do not express tyrosinase activity; and (c) we have been able to regulate independently the expression of tyrosinase and gp75 in melanoma cell lines (20).

Understanding the specificity of mAbs that react with melanosomal antigens will be important for sorting out the identity of these molecules. It has been proposed in a recent report by Jiménez et al. (41) that a second gene only distantly related to BBTY-1 and Pmel 34 (33), mapping to the b (brown) locus in the mouse (42), codes for a gene product with tyrosinase activity (41). Thus, it is becoming increasingly evident that tyrosinase is a member of a family of related molecules that include distinct genes and alternative transcripts of these genes (32–34, 36, 41, 42).

Summary

A distinguishing characteristic of cells of the melanocyte lineage is the expression of the melanosomal enzyme tyrosinase that catalyzes the synthesis of the pigment melanin. A tyrosinase cDNA clone, designated BBTY-1, was isolated from a library constructed from the pigmented TA99⁺/CF21⁺ melanoma cell line SK-MEL-19. Expression of BBTY-1 in mouse L929 fibroblasts led to synthesis and expression of active tyrosinase, and, unexpectedly, to stable production of melanin. Melanin was synthesized and stored within membrane-bound vesicles in the cytoplasm of transfected fibroblasts. BBTY-1 detected a 2.4-kb mRNA transcript in nine of nine pigmented, tyrosinase-positive melanoma cell lines. Tyrosinase transcripts of the same size and abundance were detected in a subset (three of eight) of nonpigmented, tyrosinase-negative melanoma cell lines, suggesting that post-transcriptional events are important in regulating tyrosinase activity. Two melanocyte antigens, recognized by mAbs TA99 and CF21, that are specifically located within melanosomes and are coexpressed with tyrosinase activity, did not react with transfected mouse fibroblasts expressing human tyrosinase, supporting the conclusion that these antigenic determinants are distinct from the tyrosinase molecule coded for by BBTY-1.

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THE PIGMENTARY SYSTEM
PHYSIOLOGY AND PATHOPHYSIOLOGY

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THE

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SYSTEM

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FRONTISPIECE A: These two individuals illustrate the wide range of skin and hair color [Marianne
Greenwood (right) graciously shared the photographs (from her book Varför Gråter Poman?) that com-
pose this frontispiece.] B: Classical Celtic woman with blue eyes and red hair. C: Typical Scandinavian
with blue eyes. D: Native American. E: A Peruvian girl. F: Himalayan woman and child. G: A Venezue-
lan woman. H: Two teenagers from New Guinea. I: Man from the New Hebrides Islands.

CONTENTS

Contents

ix

Chapter 18

Anatomy of Pigment Cell Genes Acting at the Cellular Level

MURRAY H. BRILLIANT AND GREGORY S. BARSH

Nearly 80 years ago, studies by Sewall Wright on color inheritance in guinea pigs laid the groundwork for recognizing that mammalian melanocytes synthesize pigment of two different types, black–brown eumelanin or yellow–red pheomelanin (Wright, 1917a,c). Wright suggested that certain genes such as *Agouti* (*A*) or *Extension* (*E*) determined which of these two pigment types would be synthesized, while others such as *pink-eyed dilution* (*p*) affected the quality of eumelanin but not that of pheomelanin.

Wright's studies were based primarily on gross observations of breeding experiments. A deeper understanding of the essential difference between pigment types came from pioneering studies carried out by Elizabeth (Tibby) Russell in the 1940s, in which detailed histologic comparisons were made of mouse coat color variants that differed at key loci (Russell, 1946, 1948, 1949a,b). This work, combined with later chemical and ultrastructural investigations (Moyer, 1963), revealed that subcellular organelles that contain eumelanin, eumelanosomes, are oval-shaped with an organized internal matrix of parallel lamellae. There can be considerable variation in the number, size, and distribution of eumelanosomes along the hair shaft; eumelanic pigment granules in *pink-eyed dilution* are irregular in shape and have a shred-like appearance. By contrast, pheomelanosomes are spherical, lack an organized internal structure, exhibit little variation in morphology, and are not affected by the *p* mutation (reviewed by Silvers, 1979).

As described in Chapter 28, an important landmark in melanin research was the recognition in the 1920s that eumelanin synthesis involved oxidation of tyrosine to form an indole-based polymer, by a process now known as the Raper-Mason pathway. Because pheomelanin is soluble in dilute alkali and has a relatively high cysteine content, it was recognized to have a different chemical structure, although its exact nature is still undetermined (reviewed by Prota, 1992). Studies in the 1950s suggested that alternative amino acids might underlie differences between eumelanin and pheomelanin, but it is now clear that both pigment types are produced solely from tyrosine, and both depend on tyrosinase-catalyzed formation of dopaquinone as described in Chapter 24. Thus, diversion of dopaquinone into the pheomelanic pathway serves as the fulcrum upon which genes that control eumelanin–pheomelanin switching are balanced (Fig. 18-1). Interestingly, Sewall Wright suggested a biochemical pathway for pigment synthesis in 1917 that was based solely on genetic data (Wright, 1917a). Though details of his hypothesis later proved incorrect, an essential component—that eumelanin and pheomelanin shared both a common precursor and initial steps of oxidative metabolism—was confirmed nearly 50 years later.

Whereas the earliest studies of coat color genetics were carried out with guinea pigs as described above, mice are much better suited to breeding studies and so have provided most of the depth in mammalian pigmentation genetics (Wright, 1917b). This resource has arisen from three different sources. Many coat-color variants that previously existed among communities of mouse fanciers or wild mice were incorporated into inbred strains during the early part of this century, such as the *nonagouti* (*a*), *tobacco* (*E*^{tob} or *Mc1r*^{tob}), and the original mutant alleles at the *Agouti*, *Mc1r*, and *pink-eyed dilution* loci (reviewed in Morse, 1978; and Silver, 1995). In addition, a large number of spontaneous mutations have arisen during the propagation of inbred strains, such as the *black-and-tan* (*a*^{t}), recessive yellow (*e* or *Mc1r*^{e}), and *pink-eyed unstable* (*p*^{un}) alleles. Finally, many additional alleles of *Agouti* and *pink-eyed dilution* have arisen from large-scale mutagenesis studies carried out at national laboratories such as Harwell (Lyon *et al.*, 1992) and Oak Ridge (Russell & Russell, 1992).

CURRENT CONCEPTS

Overview

In the last decade, molecular isolation of mouse coat-color genes and characterization of their protein products has built on the foundation laid by previous genetic and biochemical investigations to provide a deeper understanding of pigmentation biology (reviewed in Jackson, 1994). These studies have revealed that *Agouti* and *Proopiomelanocortin* (*Pomc*) encode secreted signaling molecules, agouti protein (ASP) and α-MSH, respectively, that affect melanocytes in opposite ways via a G$_s$ protein–coupled receptor encoded by *Extension*, now known as the *Melanocortin 1 receptor* (*Mc1r*) gene (reviewed in Barsh, 1996). The level of Mc1r signaling and subsequent adenylate cyclase activation determines whether pheomelanin or eumelanin are synthesized, by stimulating diversion of dopaquinone into a cysteine-derivative in the case of pheomelanin, and by activation of at least four specific genes in the case of eumelanin (Fig. 18-1). Some of the eumelanin-specific genes encode enzymatic components of the Raper-Mason pathway; these are described further in Chapter 28. However, an important component of eumelanin that does not appear to play an enzymatic role is the twelve membrane–spanning protein encoded by the *pink-eyed dilution* gene. Although the exact function of the P protein has not been determined, its predicted structure is homologous to a family of transporter proteins (Lee *et al.*, 1995).

Mouse coat-color genes have played an important role in understanding basic aspects of mammalian genetics. For example, the original *p* mutation and a mutation of the tyrosinase gene (*c* locus) were used to define the first genetic linkage group in the

217
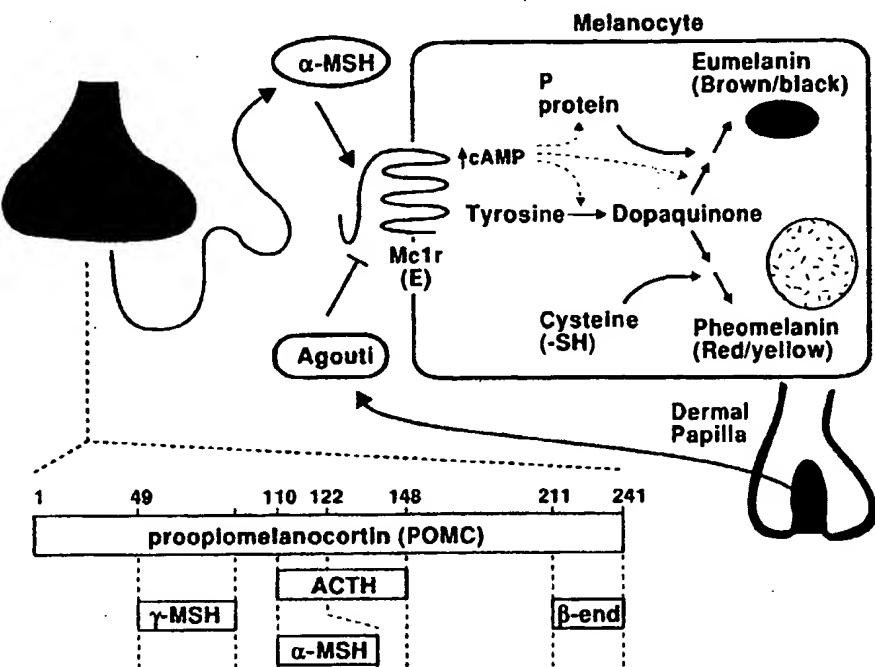
Melanocyte

Fig. 18–1. Gene products that control pigment-type switching. The polypeptide proopiomelanocortin (POMC) is cleaved by subtilisin-like proteins into several smaller neurendocrine peptides, including γ-melanocyte stimulating hormone (γ-MSH), α-melanocyte stimulating hormone (α-MSH), adrenocorticotrophic hormone (ACTH), and β-endorphin (β-end). The intermediate lobe of the pituitary gland mainly produces α-MSH, but α-MSH is also synthesized widely in the brain where it modulates a variety of neuroendocrine processes (reviewed in O'Donohue & Dorsa, 1982). In addition, as described in the text, recent studies suggest that α-MSH and/or ACTH are produced by keratinocytes and therefore may affect pigmentation as paracrine hormones. α-MSH and the Agouti protein act to stimulate and inhibit, respectively, signaling through the melanocortin 1 receptor (Mc1r), encoded by a gene previously known as *Extension* (*E*). Unlike α-MSH, which is found at physiologic levels in the bloodstream. Agouti protein does not circulate and instead is produced locally by cells in the dermal papilla. Increased signaling through the Mc1r causes an elevation of cAMP levels and likely stimulates expression of the P protein, which forms an essential component of eumelanin, but is not required to produce pheomelanin. Increased Mc1r signaling also stimulates expression of tyrosinase and other genes required for eumelanogenesis as described in Chapter 30.

mouse (Haldane *et al.*, 1915), now assigned to mouse chromosome 7 (Brilliant *et al.*, 1996). The *p* locus was also used as a marker in transplantation studies to define histocompatability antigens (Snell & Stevens, 1961). An unusual *Agouti* allele, *lethal yellow* (*Aʸ*), is caused by a deletion that simultaneously (*l*) removes an hnRNP-like gene required for RNA metabolism expressed at high levels in unfertilized oocytes, and (2) causes ubiquitous expression of agouti protein-coding sequence, leading to pleiotropic effects that include obesity, diabetes, and increased tumor susceptibility (Duhl *et al.*, 1994a; Michaud *et al.*, 1993). Segregation analysis of *Aʸ/a* intercrosses in which the ratio of *Aʸ* to non-*Aʸ* progeny was 2:1 rather than the expected 3:1 provided the first evidence of an embryonic lethal mutation in animal genetics (Cuénot, 1908), and efforts to understand the mechanism of *Agouti*-induced obesity have led to increased appreciation for the role of melanocortin signaling in intermediary metabolism and energy balance (reviewed in Yen *et al.*, 1994).

Lower Vertebrates and Invertebrates

Much of our current understanding of melanocortin signaling is based on studies carried out on hormonal control of pigmentation in amphibians and reptiles (Lerner, 1993). Melanin-containing cells in these animals, melanophores, exhibit rapid changes in their absorption of visible light mediated by the intracellular dispersion or aggregation of microscopic pigment granules. This phenomenon underlies the ability of many reptiles and amphibians to adapt to their environmental background and is mediated by a variety of different receptors for catecholamines, serotonin, and peptide hormones. Recognition that extracts of mammalian pituitary glands could reproduce this process led to the isolation of α-melanocyte stimulating hormone (α-MSH), a 13 amino acid peptide produced by proteolytic cleavage from a multifunctional larger precursor, proopiomelanocortin (Pomc) (reviewed in Bertagna, 1994; De Wied and Jolles, 1982; and Eberle, 1988).

Besides α-MSH, Pomc also gives rise to γ-MSH, adrenocorticotrophic hormone (ACTH) and β-endorphin (Fig. 18–1). α-MSH, γ-MSH, and ACTH each cause pigment dispersion in melanophores, presumably via one or more melanocortin receptors similar to those isolated in mammals (see below).

Melanocortin signaling is extremely well conserved among distantly related vertebrates. The primary sequence of α-MSH in most amphibians and fish is identical to that of mammalian α-MSH, although some differences exist in post-translational modification (N-terminal acetylation and C-terminal amidation). Biochemical studies suggest that the pale ventral color characteristic of several frog species is caused in part by production of paracrine factors in ventral skin that inhibit melanocortin signaling (Fukuzawa & Bagnara, 1989; Kreutzfeld et al., 1989). This provides an interesting parallel to mammalian Agouti, which is thought to cause the yellow or cream-colored ventral pelage observed in many species of mammals (Vrieling et al., 1994).

The pigment found in dark-colored melanophores is derived from tyrosine and is often described as eumelanin. By contrast, bright-colored pigment cells of cold-blooded vertebrates, iridophores, or xanthophores, contain pigment derived from purines or pteridines, respectively, and they do not have mammalian counterparts (Bagnara, 1983). Pheomelanin is not thought to exist in amphibians, fish, or reptiles, but it has been extensively characterized in red feathers of New Hampshire hens (reviewed in Prota, 1992). In fact, much like the mouse p gene, the chicken locus "pinkeye," pk, causes a severe reduction in retinal pigmentation, produces gray feathers in strains of wild-type birds that produce black feathers, and shows histologic similarities to the melanocytes of p/p mice (Brumbaugh & Lee, 1975). Despite these phenotypic similarities, molecular homologues of Agouti and p have only been described in mammals. An avian melanocortin receptor has been isolated recently (Takeuchi et al., 1996), and it will be of interest to determine whether altered signaling through this receptor induces pheomelanogenesis as it does in mammals (see below).

Mammals

Agouti and Extension

Populations of wild mice frequently carry the *light-bellied Agouti* (A^w) allele, in which each dorsal hair has a subterminal band of yellow on a black or brown background, while all ventral hair is yellow or cream-colored. The banded appearance of dorsal hairs gives a brushed or golden appearance to the coat and is especially prominent in a South American rodent for which the gene is named (reviewed in Barsh, 1996; and Siracusa, 1994). The yellow band is caused by hair-follicle melanocytes switching from eumelanin to pheomelanin synthesis and back again during the early anagen phase of hair growth. Some *Agouti* alleles such as *lethal yellow* (A^y) or *viable yellow* (A^{vy}) produce a coat that contains only pheomelanin, while others such as *nonagouti* (a) or *extreme nonagouti* (a^e) produce only eumelanin. When different *Agouti* alleles are compared with each other, synthesis of pheomelanin appears dominant to that of eumelanin. For example, the *lethal yellow* (A^y) and *viable yellow* (A^{vy}) alleles of *Agouti* are dominant to the a allele; e.g., A^y/a and A^{vy}/a animals are yellow but a/a animals are black. An unusual feature of dominant *Agouti* alleles such as A^y and A^{vy} is

their extrapigmentary effects. Animals that carry these alleles are not only yellow but also develop obesity, diabetes, and an increased susceptibility to several different types of tumors (reviewed in Yen et al., 1994).

Extension, named for the ability of certain alleles to extend the area of black pigmentation, is recognized in mice as four different variants; *sombre* (E^{som}), *tobacco* (E^{tob}), wild-type (E), and *recessive yellow* (e). Animals homozygous for e have a coat that contains almost exclusively pheomelanin and therefore appear very similar to animals that carry A^y. Animals that carry the E^{som} allele have coats that contain almost exclusively eumelanin in their coat. In contrast to the *Agouti* allelic series, synthesis of eumelanin is dominant to that of pheomelanin for *Extension* alleles, e.g., E^{som}/e animals are black but e/e animals are yellow.

Genetic interaction studies show that, in general, the pigmentary effects of *Extension* alleles are epistatic to those of *Agouti*. For example, the coat of an a/a animal in the presence of the wild-type E allele, i.e., a/a E/E, contains eumelanin, but the coat of an a/a e/e animal contains pheomelanin (Lamoreux & Russell, 1979). Likewise, whereas the coat of an A^y/a E/E animal contains pheomelanin, that of an A^y/a $E^{som}/-$ animal contains eumelanin. These findings point to a pigment control pathway in which *Agouti* encodes an upstream component promoting pheomelanin synthesis, whereas *Extension* encodes a downstream component promoting eumelanin synthesis (Geschwind et al., 1972; Jackson, 1994; reviewed in Silvers, 1979). This notion is also consistent with the results of skin transplantation experiments in which the *Agouti* gene was found to act in the dermal component of hair follicles while the *Extension* gene was found to act in melanocytes (Lamoreux & Mayer, 1975; Silvers, 1958, 1961; Silvers & Russell, 1955).

Molecular isolation of the *Agouti* gene, made possible by an unusual chromosomal rearrangement that fused sequences from an *Agouti* intron to those from the previously isolated *limb deformity* gene, revealed a 131 amino acid protein with an amino terminal signal sequence, a basic central region that contains a single glycosylation site, and a cysteine-rich carboxyl terminus (Bultman et al., 1992; Miller et al., 1993). Dominant *Agouti* mutations associated with pleiotropic effects such as obesity and increased tumor susceptibility such as A^y or A^{vy} were found to be caused by regulatory abnormalities leading to ubiquitous and continual expression of a normal agouti protein (Duhl et al., 1994b; Michaud et al., 1994; Miller et al., 1993; Siracusa et al., 1995). By contrast, loss-of-function mutations such as a or a^e were found to be caused either by regulatory abnormalities leading to decreased expression, in the case of a (Bultman et al., 1994), or by structural abnormalities of protein-coding sequence, in the case of a^e (Hustad et al., 1995). These findings, as well as the ability of an *Agouti* cDNA to cause production of a yellow coat when expressed as a transgene (Perry et al., 1995), confirmed the prediction that *Agouti* functioned as a positive regulator of pheomelanin synthesis. As suggested by earlier transplantation experiments, *Agouti* mRNA was found to be expressed in the dermal papillae of hair follicles, at or just prior to the time of pheomelanin synthesis (Millar et al., 1995). Thus, in animals carrying the A^w allele, *Agouti* RNA is expressed in dorsal skin for a 2- to 3-day period during anagen, coinciding with deposition of pheomelanin in the subapical region of each dorsal hair (Vrieling et al., 1994). However, in ventral skin of an A^w/A^w animal, *Agouti* RNA is expressed during the entire hair growth cycle. These observations helped to confirm the notion originally suggested from transplantation studies (Silvers

& Russell, 1955) that agouti protein has a small sphere of action (several cell diameters) and therefore acts normally as a paracrine hormone.

Agouti protein does not exhibit significant primary sequence similarity to other cDNA or protein sequences, but the spacing between carboxy-terminal cysteine residues is nearly identical to that observed in a group of invertebrate toxins that function as calcium channel antagonists. This observation has implications for the three-dimensional structure of agouti and has led to speculation about possible biochemical mechanisms of agouti signaling (Manne et al., 1995; Zemel et al., 1995). However, most evidence to date suggests that agouti protein does not act directly on calcium channels but instead antagonizes Mc1r signaling (see below).

In contrast to Agouti, whose isolation was dependent on previously existing mouse coat color mutations, proof that allelic variation in the Mc1r gene caused Extension mutations came after the Mc1r gene was isolated using an approach based on sequence similarity to other G protein–coupled receptors (Mountjoy et al., 1992). Consideration of the Mc1r as a candidate for Extension was based in part on previous studies showing that, in A^y/a hair bulb melanocytes, production of eumelanin could be stimulated by treatment with dibutyryl cAMP or α-MSH, while in e/e hair bulb melanocytes, production of eumelanin could be stimulated by dibutyryl cAMP but not by α-MSH (Tamate & Takeuchi, 1964). This suggestion was confirmed by finding that the e mutation is caused by a frameshift leading to premature truncation, and the E^{tob}, E^{som}, and $E^{som \, 3J}$ mutations are caused by missense mutations leading to the production of a hyperactive Mc1r in the case of tobacco, or a constitutively active Mc1r in the case of sombre (Robbins et al., 1993).

Both genetic and biochemical studies (described further below) demonstrate that interactions between agouti protein and the Mc1r play an important role in the normal control of pigment synthesis. Surprisingly, the same is not true of the Mc1r ligand, α-MSH. Initial studies of α-MSH biosynthesis and possible effects on pigmentation focused mainly on α-MSH as a possible endocrine hormone produced by the pituitary gland. Exogenous administration of α-MSH causes a switch from pheomelanin to eumelanin synthesis in A^y/a mice (Geschwind, 1966), and increased pituitary-derived α-MSH and/or ACTH are responsible for the darkening of skin that occurs in adrenal insufficiency (Beamer et al., 1994). However, plasma levels of α-MSH do not correlate with alterations in coat color that occur when certain strains of mice carrying A^vy darken with age, and hypophysectomy does not cause pheomelanogenesis in nonagouti mice (Geschwind et al., 1972; Thody et al., 1983). Thus circulating α-MSH probably does not act as an endocrine hormone in physiologic control of pigment synthesis in the mouse. More recently, a potential role for α-MSH in pigment synthesis has focused on a paracrine or autocrine role (reviewed in Pawelek, 1993; Slominski et al., 1993; and Wintzen and Gilchrest, 1996), which is based on the finding that expression of Pomc RNA can be detected in keratinocytes and melanocytes (Farooqui et al., 1993; Slominski et al., 1992, 1995). In most of these studies, it has been difficult to determine whether the RNA detected actually gives rise to physiologically significant levels of α-MSH. However, recent work from Lugar and colleagues has demonstrated that biologically active α-MSH and ACTH are produced by cultured keratinocytes in vitro (Schauer et al., 1994).

From a genetic perspective, it is interesting to note that chromosomal mapping studies do not suggest Pomc as a candidate for

any of nearly 100 mouse coat-color mutations (Jackson, 1994; Silvers, 1979). A null Pomc mutation could be lethal because of loss of ACTH and β-endorphin. However, if endocrine or paracrine secretion of α-MSH regulated Mc1r activation normally, hypomorphic Pomc alleles might be expected to cause a pigmentary phenotype, since small increases in plasma α-MSH levels have dramatic effects on coat color (Geschwind et al., 1972).

The biochemical process by which agouti protein causes pheomelanogenesis has garnered much interest, not only from pigment cell biologists but also from scientists interested in understanding the mechanism of agouti-induced obesity in mice that carry one of the dominant Agouti alleles such as A^y or A^vy that have pleiotropic effects (Manne et al., 1995; Yen et al., 1994). As described below, most evidence to date suggests that agouti acts as a melanocortin antagonist, preventing the binding and activation of the Mc1r by α-MSH. Genetic studies indicate that A^y-induced obesity is not mediated by the Mc1r since it is not suppressed by the e mutation. However, there are four additional melanocortin receptors expressed in a multiplicity of cell types (Gantz et al., 1993a,b, 1994), and agouti antagonism at one or more of these receptors could easily have pleiotropic effects.

In cell culture, recombinant purified agouti protein inhibits activation of adenylate cyclase elicited by α-MSH or other melanocortins and, at similar concentrations, also inhibits melanocortin binding as measured with I^{125} NDP-MSH (Blanchard et al., 1995; Lu et al., 1994; Siegrist et al., 1996; Willard et al., 1995). In most experiments, agouti protein by itself has had no effect on basal levels of cAMP accumulation or melanogenesis, or on the maximal level of Mc1r activation. These findings suggest a mechanism of competitive antagonism whereby agouti protein and α-MSH bind to the Mc1r in a mutually exclusive manner, with a calculated K_D for agouti protein of 0.5–1 nM (Blanchard et al., 1995). However, a direct interaction between agouti protein and the Mc1r has not yet been demonstrated. In addition, some experiments have demonstrated that agouti protein may cause downregulation of the Mc1r, a finding opposite to that expected for a competitive antagonist (Siegrist et al., 1996).

Other studies have suggested that agouti protein may have effects in addition to melanocortin antagonism, since its addition to mouse melanocytes or melanoma cells can stimulate pheomelanogenesis and/or depress tyrosinase activity in the absence of exogenous α-MSH (Hunt & Thody, 1995). The idea that agouti may function as a calcium channel antagonist in some cell types, including those responsible for obesity and diabetes in mice that carry A^y or A^vy, has garnered support from the observation that prolonged exposure of skeletal myocytes to agouti protein causes a rise in intracellular calcium, and that skeletal tissue from insulin-resistant A^vy/a animals exhibits elevated levels of intracellular calcium (Jones et al., 1996; Zemel et al., 1995). However, a primary effect of agouti protein on calcium channels has not yet been demonstrated.

Region-specific regulation of Agouti gene expression

One of the most interesting aspects of the Agouti gene is that its effects are confined to specific parts of the body in different alleles (Plate 10). Whereas mice that carry the light-bellied Agouti (A^w) allele have dorsal hairs that are banded and ventral hairs that are completely yellow or cream-colored, mice that carry the Agouti (A) allele have banded hairs on dorsum and ventrum, and mice that carry the black-and-tan (a^t) allele have dorsal hairs that are black and ventral hairs that are yellow.

Molecular cloning studies have now revealed that these observations are explained by the complex genetic structure of the *Agouti* locus in which two different regulatory regions simultaneously control expression of protein-coding sequences and that each regulatory region can be mutated independently (Bultman *et al.*, 1994; Chen *et al.*, 1996; Vrieling *et al.*, 1994).

As shown in Plate 10, one *Agouti* promoter directs expression of protein-coding sequences throughout the entire hair-growth cycle but affects only ventral skin, while a second promoter directs expression in both dorsal and ventral skin, but only in the mid-phase of the hair growth cycle (Vrieling *et al.*, 1994). In the wild-type A^w allele, both promoters are active to yield the light-bellied agouti phenotype. In the *A* allele, intrachromosomal crossing over mediated by an inverted duplication inactivates the ventral-specific promoter to produce the agouti phenotype (Chen *et al.*, 1996). Finally, in the a' allele, insertion of a retroviral-like element inactivates the hair-cycle promoter to produce the black-and-tan phenotype (Bultman *et al.*, 1994). In a'/a' animals, pheomelanin is produced not only in the ventral trunk but also in specialized areas of skin that surround the external ear and the dorsomedial aspect of the distal limbs, which suggests that the "ventral-specific" *Agouti* promoter is only ventral-specific in the trunk, and that it also controls localized expression in other areas of the body. This observation is particularly relevant in considering how coat-color patterns in mammals other than mice might be explained (see below).

The pink-eyed dilution gene

The original *p* allele, which probably arose in Manchuria or Japan in *Mus molosinus* (Brilliant *et al.*, 1994a), was incorporated into several common laboratory strains of mice (e.g., SJL/J, 129/J, P/J, and FS/Ei) during the early part of this century. The first mutant allele of the *p* locus to be characterized molecularly was the p^{un} (*pink-eyed unstable*) allele, which exhibits high-frequency (somatic) reversion to wild-type (Melvold, 1971). Using the technique of genome scanning, the p^{un} allele was found to result from a duplication of 75 kb of genomic DNA (Brilliant *et al.*, 1991; Gondo *et al.*, 1993), now known to include exons 6–18 (Oakey *et al.*, 1996) of the 24-exon *p* gene. A fragment of genomic DNA that cross-hybridized to various mammalian species (now known to include exon 19) was used to screen a melanoma cDNA library to obtain the *p* gene cDNA and its human homologue (Gardner *et al.*, 1992). Parallel studies using a candidate gene approach found that a previously unknown human cDNA fragment (DN10) identified an alternative exon of the human *P* gene (Lee *et al.*, 1995; Rinchik *et al.*, 1993). Many mouse mutant *p* alleles have now been characterized at the molecular level (Culiat *et al.*, 1993, 1994; Gardner *et al.*, 1992; Johnson *et al.*, 1995; Nakatsu *et al.*, 1993; Oakey *et al.*, 1996; Rinchik *et al.*, 1993). The discovery of the human *P* cDNA has led to an understanding of the molecular basis for tyrosinase positive oculocutaneous albinism (OCA2) discussed below and in Chapter 38.

Mouse *p* alleles and phenotypes

To date, more than 100 *p* alleles have been identified, some of which were *de novo* in origin and some of which were induced by Xrays or chemical mutagens (Lyon *et al.*, 1992; M. F. Lyon, personal communication; Russell *et al.*, 1995). In the homozygous state, each mutant *p* allele causes hypopigmentation ranging from

a minor reduction in coat color to a dramatic reduction of both coat and eye color characteristic of the original *p* mutation. In addition to affecting pigmentation, several mutant alleles are associated with other abnormalities, including decreased neonatal viability, neurological disorders, cleft palate, male sterility, female semi-fertility, viability, and prenatal lethality (Brilliant, 1992; Culiat *et al.*, 1993, 1994; Lyon *et al.*, 1992; Nakatsu *et al.*, 1993; Russell *et al.*, 1995). All of the mutations with these additional phenotypes were induced by radiation and affect surrounding genes (Johnson *et al.*, 1995; Lyon *et al.*, 1992; Russell *et al.*, 1995). However, mutations of the *p* gene alone cause effects on pigmentation only (Gardner *et al.*, 1992; Johnson *et al.*, 1995; Lyon *et al.*, 1992; Russell *et al.*, 1995).

The pigmentation defects associated with *p* mutations have been characterized in detail. The reduction in pigmentation is the result of a reduction in melanin (primarily eumelanin). In the hair, *p* pigment granules have been described as shred-like (Russell, 1949a). The same description has been applied to the small, irregular-shaped melanosomes in the Harderian gland of *p* mice (Markert & Silvers, 1956). In *p* mice, premelanosomes from embryonic choroid and retina are fibrillar in appearance, as are those from the adult choroid. However, most melanosomes from the adult retina are more particulate in appearance. Most *p* melanosomes show some pigment indicated as an increase in electron density and diameter of melanofilaments, but none are fully melanized (Hearing *et al.*, 1973). The melanosomes within *p/p* melanocytes appear as stage 1 and stage 2 melanosomes, as described in the above references (Fig. 18–2). Transplantation studies in the mouse have demonstrated that the *p* defect is intrinsic to melanocytes (Stephenson & Hornby, 1985).

The p gene protein and speculation about its function

Recent molecular analysis of the p transcript and protein have corroborated much of the past phenotypic data and have extended our knowledge about the p protein's role in pigmentation, even if its exact function is unknown at this time. Northern blot analyses have confirmed that melanocytes are the predominant p gene-expressing cell type, with low-level expression of the p gene in brain, testes, and ovaries. The size of the mouse p gene transcript is 3.3 kb, encoding a predicted protein of 833 amino acids (Gardner *et al.*, 1992; Rinchik *et al.*, 1993). The mouse p gene product, like its human homologue *P*, encodes a protein with 12 membrane-spanning domains. Using antisera against a synthetic peptide from the first luminal loop (amino acids 285–298), Rosemblat *et al.* (1994) were able to characterize the p protein as an integral, melanosomal membrane protein of 110 kDa that does not appear to be glycosylated (tunicamycin treatment does not alter its gel mobility).

There is evidence that the p protein interacts with melanin. The p protein (along with the silver protein) is far less extractable from melanized melanosomes (in melanocytes or in a cell-free assay system) than it is from poorly melanized melanosomes (Donatien & Orlow, 1995). It may be that some of the loops of the p protein that protrude inside the melanosome are somehow trapped within the melanin polymer. If this close association with melanin impedes p protein function, then perhaps this is one way to limit the melanin content of the developing melanosome. An interesting observation is that the *p* transcript is expressed in the black dorsal skin, but not the yellow ventral skin in a'/a' mice (Rinchik *et al.*, 1993), corroborating earlier notions that the *p* mutations affect eumelanin but not
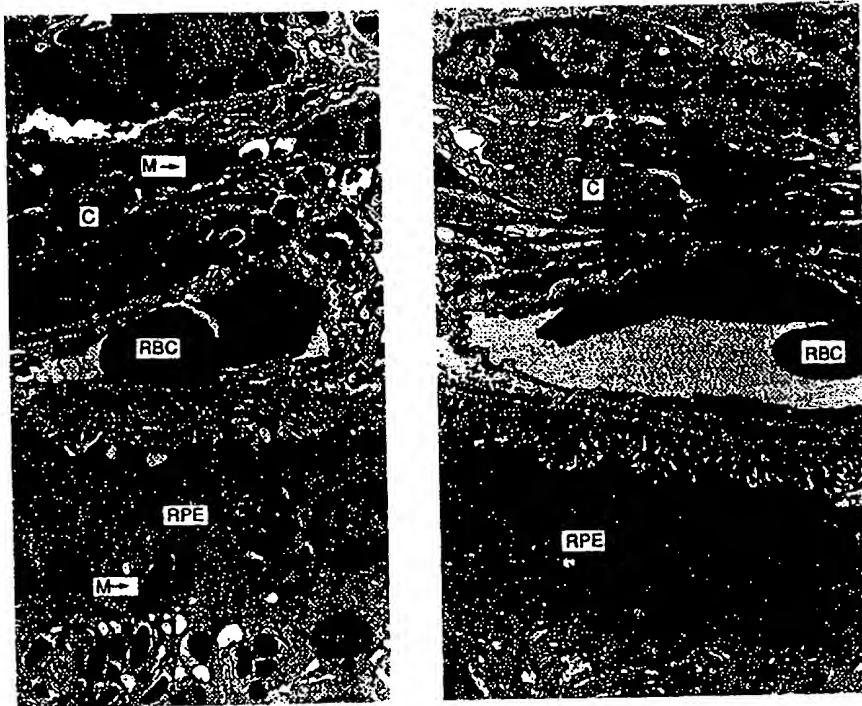
Fig. 18–2. A comparative study of eye tissue from wild-type and *pink-eyed dilution* mice. A cross section of an eye of a wild-type mouse (left) is shown in comparison to one from a mutant (*p^un^/p^un^*) mouse (right). Note the lack of mature melanosomes (M) in the *p^un^/p^un^* mouse eye in both the choroid (C) and the retinal pigmented epithelial layer (RPE). Also shown are red blood cells (RBC) within blood vessels.

pheomelanin biosynthesis. Thus, the p protein may play a role in the eumelanin–pheomelanin switch.

From its predicted protein structure, the p protein may be a transporter critical to melanocyte function (Gardner et al., 1992; Rinchik et al., 1993). It has been proposed that the p protein is a tyrosine transporter (Rinchik et al., 1993), based on sequence homology that has been subsequently shown not to be significant (Lee et al., 1995; Rosemblat et al., 1994). Supporting the hypothesis, however, are the observations that both mouse and human melanocytes can be induced to pigment in vitro with high tyrosine (Sidman & Pearlstein, 1965; Witkop et al., 1973). However, recent direct biochemical assays show no difference in tyrosine transport between normal and p melanocytes at the level of the melanocyte or the melanosome. Plasma membrane tyrosine transport was found to be normal in p (p^cp^/p^cp^) melanocytes (K_m 89 μM; V_max 302 pmol/min/mg cell protein), and the melanosome-rich granular fractions of normal (melan-a) and p melanocytes (p^cp^/p^cp^) were essentially the same, taking up 10 μM [³H] tyrosine at about 21 pmol/min/mg protein (Gahl et al., 1995). Thus, although the p protein may be a transporter, it does not significantly contribute to tyrosine transport. The proteins most homologous to p are bacterial and include the S. aureus and E. coli ArsB proteins (the anion-conducting pathway of a group of proteins that together confer resistance to arsenic), the E. coli Na⁺/H⁺antiporter, and protein 38L from M. leprae (Lee et al., 1995; Rosemblat et al., 1994). It may be that the p protein is similarly involved in ion transport, potentially mediating the low pH of the melanosome that favors the enzymatic action of tyrosinase. An alternative hypothesis is that the p protein functions to transport sulfhydryl compounds

out of the melanosome to permit the formation of eumelanin (Lamoreux et al., 1995). Indeed the p protein has been hypothesized to affect the rate of an existing melanin-synthesizing enzyme system to achieve normal pigmentation (Coleman, 1962; Sidman & Pearlstein, 1965). Other observations suggest that the p, tyrosinase, and TRP-1 (tyrosinase related protein-1) proteins exist in a high molecular weight complex that is not formed in p mutant melanocytes (Chiu et al., 1993; Lamoreux et al., 1995). Thus, the p protein may also play a coordinating role in melanogenesis.

The p protein may be specifically targeted to the melanosome or one of its precursors. In normal pigmented melanocytes, a fraction of the p protein is present in an intracellular compartment distinct from that containing tyrosinase and TRP-1. It has also been noted that melanosomes lacking p protein are missing a high molecular weight complex of the p protein, tyrosinase and TRP-1, and they possess characteristics of immature premelanosomes (Rosemblat et al., 1994). Thus, in addition to a potential transport function, the p protein may also play a critical role in the biogenesis of normal melanosomes, perhaps by providing conditions (e.g., low pH) favorable for their proper structure and maturation. The melanosome, as a specialized endosome, may derive its protein components by means of signals and sorting mechanisms that distinguish it from a lysosome. In fact, a specific protein sequence motif, capable of targeting TRP-1 to the melanosomal membrane, exists within its carboxyl-terminus (Vijayasaradhi et al., 1995). When this TRP-1 amino acid sequence was incorporated in chimeric proteins, they were correctly targeted. The sequence capable of targeting TRP-1 is conserved across species and a related sequence is found in several

other melanosome proteins, including the p protein. However, it remains to be shown whether the sequence in the p protein related to the TRP-1 sorting sequence actually functions in the same way. If so, it would confound the observation that a subset of the p protein is in a different intracellular compartment from tyrosinase and TRP-1 (Rosemblat et al., 1994).

Evolutionary considerations

There is substantial evidence for conservation of the *Agouti*, *Extension*, and *pink-eyed dilution* genes in different classes of mammals including ungulates, lagomorphs, canines, marsupials, and primates (see below). The coat-color patterns of light-bellied agouti and black-and-tan are prevalent among certain orders; in larger animals with a black-and-tan phenotype, e.g., German shepherds or Doberman pinschers, pheomelanin markings are also evident on the distal limbs and areas of the head and neck (Little, 1957; Searle, 1968; Willis, 1989). These phenotypes and others that involve localized differences in the distribution of pheomelanin and eumelanin have for the most part been attributed to allelic variation at *Agouti* or *Extension* (Adalsteinsson et al., 1987, 1994, 1995). While in most cases such assumptions are probably correct, few have yet been proven at a molecular level. Most inferences regarding homology are based on dominance relationships for a particular allelic series and/or interaction between alleles of different loci. Thus, alleles associated with pheomelanin production should be dominant to those associated with eumelanin production for *Agouti*, the opposite should be true for *Extension*, and, in animals in which dominant and recessive alleles exist for both genes, *Extension* should be epistatic to *Agouti*. An oft-cited exception to these precepts exists for canines, in which a presumptive *Agouti* allele associated with eumelanin production, *A*, is dominant to one associated with pheomelanin production, *a^y* (Willis, 1989). However, it can be difficult to distinguish epistasis from dominance, and the canine *A* mutation may instead be an *E^som*-like allele of *Extension*.

For *pink-eyed dilution*, mice, deermice, rats, rabbits, and cats all have an equivalent locus defined by a tyrosinase-positive, OCA2-like phenotype and which is part of a conserved linkage group in these species (Heim et al., 1988; reviewed by Little, 1958). A naturally occurring variant of the black bear, the Kermode bear, found in British Colombia, is associated with a recessive *p*-like mutant phenotype (Allen, 1909). In many mammalian species, the conserved linkage group includes the β-globin gene cluster. However, in humans the β-globin locus (on human chromosome 11) is not linked to OCA2 (Heim et al., 1988), which is now known to be associated with mutations of the *P* gene on chromosome 15 (Durham-Pierre et al., 1994; Lee et al., 1994a,b; Rinchik et al., 1993), described below. Southern hybridization using a fragment of the mouse *p* cDNA as a probe confirms the widespread conservation of the *p* gene in mammals (Fig. 18-3).

Other genes that affect eumelanin and pheomelanin

Besides *Agouti* and *Extension*, mutations of three mouse coat-color genes, *mahogany* (*mg*), *mahoganoid* (*md*), and *Umbrous* (*U*), suppress *Agouti*-induced pheomelanogenesis in much the same way as dominant *Extension* alleles. Although the molecular identities of *mg*, *md*, and *U* are not known, homologues are likely to exist in other mammals and may represent additional genetic
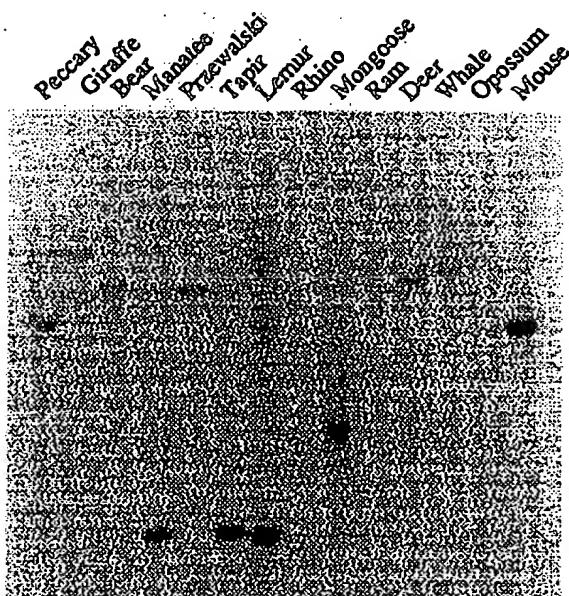
Fig. 18–3. Conservation of *pink-eyed dilution* gene. Southern hybridization using a fragment of the mouse *p* cDNA as a probe confirms the widespread conservation of the *p* gene in the mammalian species indicated.

controls over the distribution and deposition of pheomelanin versus eumelanin. Finally, two other genes, *Tabby* and *Sex-linked orange*, are important determinants of pheomelanin–eumelanin-based coat-color patterns in many carnivores, but homologs have not been identified in rodents (Robinson, 1991; Searle, 1968). Alleles of *Tabby* such as *mackarel* (*T*) and *blotched* (*tb*) suppress *Agouti*-induced pheomelanogenesis, but in contrast to *mg*, *md*, *U*, and *E^som*, which affect the entire body, the effects of *Tabby* alleles are manifest in a pattern of broad stripes or spots. Because the dominant *Tabby* allele in cats, *Abyssinian* (*T^a*), has no effect on the agouti band, *Tabby* is more analogous to *mg* or *md* than it is to *U* or *E^som* (Lomax & Robinson, 1988). Variation in *Tabby* gene action is thought to explain not only domestic Tabby cat but also the stripes, spots, and geometric patterns typical of tigers and leopards (Searle, 1968; Weigel, 1961). *Sex-linked orange* has been well characterized in domestic cats; homozygotes or hemizygotes have pheomelanic coats while heterozygous females exhibit fine stripes (more dense than *Tabby*), as typified by a tortoiseshell cat. The *Sex-linked orange* mutation is epistatic to *nonagouti* and therefore somewhat analogous to *recessive yellow*, but it would be extremely unusual to find that the *Mc1r* gene is responsible for *Sex-linked orange* since there are almost no exceptions to the conservation of X linkage among eutherian mammals (Ohno's law).

Human

Human homologs of *Agouti*, *Extension*, and *pink-eyed dilution* have all been identified and characterized at the molecular level and are known as *ASIP*, *MC1R*, and *P*, respectively. Mutations of the human *P* gene are responsible for type II oculocutaneous albinism (OCA2). Polymorphic variation in the human *MC1R* is

likely to be partially responsible for fair skin and red hair, whereas a function has not yet been identified for the *ASIP* gene.

Agouti and *Extension*

Although humans and higher primates lack agouti-banded hairs typical of most other mammals, differential synthesis of pheomelanin and eumelanin account for a considerable amount of phenotypic variation in human pigmentation (Ito, 1993; Ito & Fujita, 1985; Thody *et al.*, 1991). Black, brown, and blond hair is composed mostly of eumelanin; but carrot-red hair contains almost exclusively pheomelanin. Therefore, by analogy to mice and other mammals, one would anticipate that loss-of-function alleles in the *MC1R* and/or gain-of-function alleles in *ASIP* might be partially responsible for red hair and fair skin in humans (Barsh, 1996; Wilson *et al.*, 1995). Evidence supporting this notion for the *MC1R* has come from recent studies of a population of Irish and British Caucasians, in whom red hair and fair skin were strongly associated with homozygosity or compound heterozygosity for one of nine sequence variants of the *MC1R* (Valverde *et al.*, 1995). While the implications of this observation—reduced activity of the *MC1R* variants and semidominant transmission—have not yet been verified, it seems likely that the *MC1R* will be one of several loci that helps to determine human red hair and fair skin, at least in Western European Caucasoids.

Albinism, OCA2, and the *P* gene

Oculocutaneous albinism (OCA) is characterized by abnormally low amounts of melanin in the eyes and skin (King *et al.*, 1994; see Chapter 38). Abnormally low amounts of melanin in the developing eye lead to abnormal routing of optic nerve fibers resulting in strabismus and loss of binocular vision. Other ocular features of albinism include photophobia, nystagmus, and foveal hypoplasia with reduced visual acuity. The reduction in skin pigmentation in individuals with OCA is associated with an increased sensitivity to ultraviolet radiation and a predisposition to skin cancer. There are two major types of OCA, tyrosinase-related OCA and tyrosinase-positive OCA, the latter being the most common form of albinism (King *et al.*, 1994). Mutations of the human tyrosinase gene on chromosome 11 lead to tyrosinase-related albinism, defined as OCA1 (Tomita *et al.*, 1989; reviewed by Oetting and King, 1993; and King *et al.*, 1994; see Chapter 38). The human *P* gene is on chromosome 15q in a region demonstrated to be linked to OCA2 in native South Africans (Kedda *et al.*, 1994; Ramsay *et al.*, 1992). Mutations in the human *P* gene lead to tyrosinase-positive OCA, defined as OCA2 (Durham-Pierre *et al.*, 1994; King *et al.*, 1994; Lee *et al.*, 1994a,b; Rinchik *et al.*, 1993). The cutaneous phenotype of OCA2 is broad, ranging from minimal to moderate pigmentation of the hair, skin, and iris. The skin pigment tends to be localized in freckles, lentigines, or nevi rather than generalized, and the ability to tan is not well defined (King *et al.*, 1994; see Chapter 38). In contrast to OCA1, individuals with OCA2 usually have pigmented hair at birth that tends to darken somewhat with age.

In the general population of the United States, tyrosinase-positive OCA occurs in 1/30,000 Caucasians and in 1/17,000 blacks (Witkop, 1985). *P* gene mutations (OCA2) have been detected in these and other racial groups. There are several genetic isolates with a very high frequency of tyrosinase positive OCA, e.g., the Brandywine, Maryland isolate (1/85) and several native North American Pueblo Indian groups: the Zuni, Hopi, and Jemez people (approximately 1/240) (Witkop *et al.*, 1972; Witkop, 1985; Woolf & Dukepoo, 1969), as well as several Native South American Indian groups (reviewed by Jeambrun and Sergent, 1991). Presumably, because these represent small restricted populations, individuals within these populations are homozygous for the same recessive mutation of a gene required for normal pigmentation. The *P* gene is a likely candidate for the OCA seen in all of these groups. Indeed, individuals with tyrosinase-positive OCA from the Brandywine, Maryland isolate are homozygous for a deletion allele of the *P* gene (a 2.7 kb deletion that includes exon 7) (Durham-Pierre *et al.*, 1994). However, it is formally possible that mutations in another gene(s) lead to a similar phenotype of tyrosinase-positive OCA in individuals and groups for which no molecular data is yet available, i.e., tyrosinase-positive OCA is itself a heterogeneous disease.

The tyrosinase-positive OCA phenotype in Africans and African-Americans is characterized by yellow hair, white skin (sometimes with localized pigmented ephelides) (Stevens *et al.*, 1995), and irises that are partially or completely pigmented (King *et al.*, 1994; see Chapter 38). This is the most common albinism with a high frequency in these populations (ranging from 1/2000 to 1/5000 in large parts of Sub-Saharan Africa). The most common mutation in this group (so far found exclusively among Africans and individuals of African ancestry) is a 2.7 kb deletion that removes exon 7 along with flanking intron sequences, first identified in the Brandywine, MD isolate (Durham-Pierre *et al.*, 1994). It is estimated that this single mutation is associated with 25%–50% of all mutant *P* alleles in African-Americans (Durham-Pierre *et al.*, 1994, 1996), although other diverse mutant alleles have been described in this population (Lee *et al.*, 1994b). The 2.7 kb deletion allele accounts for close to 80% of mutant *P* alleles in South Africa, Tanzania, and other parts of Sub-Saharan Africa (Durham-Pierre *et al.*, 1994; Spritz *et al.*, 1995; Stevens *et al.*, 1995). The phenotypic range of OCA2 is now being defined through the molecular characterization of the gene in different individuals with albinism, and it is expected that *P* gene mutations in OCA2 will be diverse. The missense mutations described to date do not seem to cluster in any specific region of the peptide, as observed for tyrosinase, but most mutations described so far are in the carboxy half of the polypeptide that contains the majority of the 12 membrane-spanning domains. A significant portion of the *P* missense mutations are found at amino acids conserved between the mouse and human *P* genes and a group of bacterial transport proteins with 12 membrane-spanning domains (Lee *et al.*, 1995).

The size of the human *P* gene transcript is 3.4 kb, encoding a predicted protein of 838 amino acids (Rinchik *et al.*, 1993). Both the human and mouse proteins encode a 12 membrane-spanning domain protein of unknown function, but related to a group of transport proteins (Gardner *et al.*, 1992; Lee *et al.*, 1995; Rinchik *et al.*, 1993; Rosemblat *et al.*, 1994). The *P* gene is encoded by 24 exons (plus one alternate exon that contains an in frame stop codon, corresponding to IR10–1, an anonymous genomic clone) that span approximately 250 to 650 kb of genomic DNA (Lee *et al.*, 1995). The human proximal promoter region contains sequences that might be binding sites for transcription factors including the following 12 motifs: 1 AP4, 4 discrete and 1 complex AP2, 1 CF1, 1 GCF, 3 SP1, and 1 TFIID. No TATA or CCAAT motifs or melanocyte-specific motifs (i.e., M box) have been described (Lee *et al.*, 1995).

The *P* gene in Prader-Willi syndrome, Angelman syndrome, hypomelanosis of Ito, and hypopigmentation

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are genetic diseases associated with chromosome 15q aberrations. PWS and AS both localize to the same chromosomal region, 15q11q13, although the critical regions for the two syndromes are distinct (reviewed by Knoll *et al.*, 1993; and Nicholls, 1993). Two common types of deletions are seen in PWS and AS patients (Christian *et al.*, 1995) and both types disrupt the marker D15S12 (IR10) within the human *P* gene. PWS involves a loss of a paternal component of 15q11q13, with or without maternal disomy for 15q. The opposite inheritance pattern is seen in AS, as it is associated with a deletion of the maternal component of 15q11q13, with or without paternal disomy for 15q.

Among the common clinical features of PWS are hyperphagia leading to obesity, mental retardation, behavior problems, craniofacial abnormalities, and hypogonadism (especially in males) leading to infertility (Bray *et al.*, 1983). The clinical features of AS include severe mental retardation, microcephaly, seizures, hypotonia, ataxia, and craniofacial abnormalities (Clayton-Smith, 1993; Magenis *et al.*, 1990). Both syndromes are also associated with hypopigmentation, as many of these patients have much lighter skin, eye, and hair color than other family members (Butler, 1989; Clayton-Smith, 1993; Hittner *et al.*, 1982; King *et al.*, 1993; Saitoh *et al.*, 1994; Weisner *et al.*, 1987). Hypopigmentation is observed in most PWS and AS patients with deletions of 15q11q13, and more specifically among those with a disruption of D15S12, identified by the IR10 probe (Hamabe *et al.*, 1991). Thus, patients who are hemizygous for the *P* gene in the context of a large deletion of 15q are hypopigmented. This observation is difficult to resolve with the recessive nature of both human *P* and mouse *p* mutations. Perhaps other genetic determinants of pigmentation are in the 15q11q13 interval. In addition to observing hypopigmentation in PWS and AS patients, several cases of PWS and AS are associated with OCA2 (Creel *et al.*, 1986; Fryburg *et al.*, 1991; Wallis & Beighton, 1989), with deletion of one homologue of the *P* gene in the context of PWS or AS and inheritance of a mutation on the other homologue (Brilliant *et al.*, 1994b; Rinchik *et al.*, 1993). Another pigmentation disorder, hypomelanosis of Ito (HI) is genetically complex, with a subset of patients being mosaic for 15q anomalies (reviewed by Pellegrino *et al.*, 1995). These HI patients have hypopigmented whorls, streaks, or patches in addition to some phenotypic similarities with PWS or AS. As in PWS and AS, the *P* gene is likely to underlie at least part of the hypopigmentation phenotype in those HI patients with 15q anomalies.

PERSPECTIVES

Biochemical Mechanisms of Pigment Type Switching

Molecular characterization of *Agouti*, *Extension*, and *pink-eyed dilution* has revealed a great deal about pigment cell biology, but the biochemical mechanisms that directly effect a switch between eumelanin and pheomelanin synthesis are still not clear. As described above, studies of the Mc1r and agouti protein suggest that increased adenylate cyclase activity is sufficient to switch from pheomelanin to eumelanin synthesis;

conversely, decreased adenylate cyclase activity is sufficient to switch from eumelanin to pheomelanin synthesis. The paradigm of cAMP-regulated gene expression, established for genes such as somatostatin and VIP (vasoactive intestinal polypeptide), points to a signaling circuit in which phosphorylation of the transcription factor CREB would activate expression of eumelanin-specific genes, including *p*, *Trp1*, *Trp2*, and *si*. However, it is not known if CREB binds directly to regulatory regions of these genes, nor whether their activation is sufficient to induce eumelanin synthesis. The phenotypic effects of mutations in *Trp1*, *Trp2*, or *si*, described in detail in Chapter 19, are relatively mild when compared with *p* mutations, which, as described above, drastically reduces the quantity of eumelanin and has little or no effect on pheomelanin.

Cessation of *p* expression is probably required for pheomelanin synthesis, but clearly it is not sufficient. An additional factor that may come into play is a quantitative alteration in the rate of dopaquinone formation. Expression of tyrosinase, encoded by the *c* gene, is reduced but not eliminated during the eumelanin-to-pheomelanin switch, leading to a commensurate decline in the production of dopaquinone. Like *p*, however, reduced tyrosinase activity may be necessary for a eumelanin-to-pheomelanin switch, but it is almost certainly not sufficient, since substitution by a series of progressively hypomorphic alleles at the *c* locus does not itself cause pigment type switching (in an *a/a E/E* background). Similarly, whereas *p* in combination with a hypomorphic *c* allele has an additive effect on coat color phenotype, animals doubly mutant for *p* and *chinchilla* (*c^ch*) exhibit dilution of eumelanin but not increased synthesis of pheomelanin.

These considerations suggest that certain genes are required for pheomelanin but not eumelanin synthesis; these genes must be activated in response to increased agouti and/or reduced Mc1r signaling but they have not been identified yet at a molecular level. Support for this idea was apparent to Sewall Wright nearly 80 years ago, when he described a coat color mutation in guinea pigs, *fading yellow* (*f*), that specifically affects pheomelanin. Finally, the mouse mutations *grey-lethal* (*gl*) and *grizzled* (*gr*) diminish pheomelanin but not eumelanin formation; these mutations also have pleiotropic effects, causing osteopetrosis and growth retardation, respectively. Thus, certain genes activated by increased agouti and/or reduced Mc1r signaling in melanocytes may also be required for vital processes in other cells. Molecular isolation of these genes and characterization of their protein products is likely to bring further understanding not only to pigment cell biochemistry but also to more general processes of mammalian development.

Summary

1. There are two major types of melanin pigment produced by melanocytes in mammals: eumelanin (black–brown) and pheomelanin (yellow–red). Both require the enzymatic oxidation of tyrosine to form dopaquinone. During pheomelanin synthesis, dopaquinone is produced at relatively low levels and becomes incorporated into sulfhydryl derivatives. By contrast, eumelanin synthesis requires a high rate of dopaquinone production and subsequent enzymatic oxidation into indole derivatives. Several genes are known to regulate whether melanocytes produce pheomelanin or eumelanin; these genes and their homologues in other species are the subject of this chapter. *Proopiomelanocortin 1* (*Pomc1*) encodes α-melanocyte stimulating hormone (α-MSH), which activates a seven transmembrane domain melanocyte receptor encoded by the *Extension* gene (recently renamed the *Melanocortin 1 receptor,*

Mclr). *Agouti* (*A*) encodes a novel paracrine- signaling molecule that antagonizes the action of α-MSH, and *pink-eyed dilution* (*p*) encodes a 12-transmembrane domain melanosomal protein whose function is unknown.

2. Many mammals have a coat-color pattern in which each dorsal hair contains a subapical band of pheomelanin on a eumelanic background, while ventral hairs almost entirely contain pheomelanin. This phenotype, light-bellied agouti, is caused by local action of the *Agouti* gene, which is expressed in specialized dermal cells and causes overlying hair-follicle melanocytes to switch from eumelanin to pheomelanin synthesis. The cell-surface receptor or receptors that agouti protein binds to have not been identified directly, but most evidence indicates that agouti protein interacts with the Mclr to prevent its activation and subsequent stimulation of adenylate cyclase. Some mutant alleles of *Agouti* are caused by regulatory abnormalities leading to ubiquitous expression of a normal agouti protein. These mutant alleles have pleiotropic effects, including a yellow coat color, obesity, diabetes, and increased tumor susceptibility.

3. Elevated levels of α-MSH cause a switch from pheomelanin to eumelanin synthesis, but genetic variants of *Pomc1* have not been recognized as determinants of coat color. By contrast, alleles of *Extension* that constitutively activate or hyperactivate Mclr function cause increased synthesis of eumelanin and decreased synthesis of pheomelanin. Conversely, an allele of *Extension* that inactivates Mclr function causes pheomelanin to be synthesized almost exclusively.

4. The *pink-eyed dilution* locus, *p*, and its human homologue, *P*, encode a protein of 110 kDa with 12 membrane–spanning domains that is localized to the melanosome membrane. The p protein is required for normal melanin (primarily eumelanin) biosynthesis. Although the exact function of the p protein is currently unknown, it has a predicted structure in common with transporter proteins. Indeed, it has been proposed that the p protein functions to transport tyrosine (the initial substrate in the melanin biosynthetic pathway). However, recent direct biochemical assays demonstrate that p mutant melanocytes and melanosomes exhibit normal tyrosine transport. Many radiation-induced alleles of the mouse *p* locus on chromosome 7 are associated with nonpigmentation phenotypes. However, the primary function of the p protein is in pigmentation. All other phenotypes are associated with deletions extending into neighboring genes.

5. Mutations of the human *P* gene on chromosome 15q11.2−q12 lead to tyrosinase positive oculocutaneous albinism, defined as OCA2, a recessive genetic disorder. OCA2 is relatively common among individuals of African origin, in whom a common haplotype is associated with a 2.7 kb deletion that includes exon 7 of the *P* gene. In some African populations this deletion allele comprises about 80% of the *P* gene mutations.

6. Deletions of the human chromosomal interval 15q11q13 cause distinct phenotypes known as Prader-Willi syndrome (PWS) or Angelman syndrome (AS), depending on whether the deletion is, respectively, paternal or maternal in origin. Although the critical regions associated with these imprinting syndromes are distinct from each other and from the *P* gene, one homologue of the *P* gene is often deleted in PWS and AS patients. Those AS and PWS patients who are hemizygous for the *P* gene (in the context of a large deletion of 15q) often exhibit hypopigmentation relative to other family members, a puzzling outcome, as carriers of null alleles of the *P* gene (in the context of a normal chromosome 15) are normally pigmented.

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Original Research Article

Effect of Arbutin on Melanogenic Proteins in Human Melanocytes

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The inhibitory effect of arbutin, a naturally occurring β-D-glucopyranoside derivative of hydroquinone, on melanogenesis was studied biochemically by using human melanocytes in culture. Cells were cultured in the presence of different concentrations of arbutin. The maximum concentration of arbutin that was not inhibitory to growth of the cells was 100 μg/mL. At that concentration, melanin synthesis was inhibited significantly by ~20% after 5 days, compared with untreated cells. This phenotypic change was associated with the inhibition of tyrosinase and DHICA polymerase activities, and the degree of inhibition was dose dependent. No significant difference in DOPAchrome tautomerase (DT) activity was observed before or after arbutin treatment. Western blotting experiments revealed there were no changes in protein content or in molecular size of tyrosinase, TRP-1 or TRP-2, indicating that inhibition of tyrosinase activity by arbutin might be due to effects at the post-translational level.

Key words: Tyrosinase, TRP-1, TRP-2, DHICA polymerase, Arbutin, Melanogenesis

INTRODUCTION

Traditionally, research on the regulation of melanogenesis has focused on factors affecting tyrosinase, which catalyzes the rate limiting step of the melanogenic pathway: the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). However, recent studies have indicated that there are additional factors affecting other steps in this pathway, including the conversion of DOPAchrome to 5,6-dihydroxy-indole-2-carboxylic acid (DHICA) by TRP-2 (tyrosinase related protein-2) as DOPAchrome tautomerase (DT), the oxidation of DHICA by TRP-1 (tyrosinase related protein-1) as DHICA oxidase; the polymerization of DHICA by pmel17/silver locus protein as DHICA polymerase, stablin, etc. (Hearing and Tsukamoto, 1991; Hearing, 1998; Chakraborty et al., 1996; Pawelek et al., 1992).

Cutaneous hyperpigmentation, including freckles and senile lentigines, is a common pigmentary disorder to humans, which becomes more prominent with aging, especially in Japanese. Arbutin (hydroquinone-β-D-glucopyranoside) is an active ingredient of the crude drug *Uvae Ursi Folium* and of certain herbs, and has been used in Japan for a long time as a chemotherapeutic agent against such pigmentary disorders (Sugai, 1992). Since arbutin has a hydroquinone moiety, it would seem likely that arbutin acts similarly to hydroquinone, which inhibits tyrosinase (Chen and Chavin, 1976; Passi and Nazzaro-Porro, 1981) and is cytotoxic to melanocytes (Jimbow et al., 1974; Penny et al., 1984; Smith et al., 1988). However, arbutin is much less cytotoxic than hydroquinone to melanocytes in culture,

and further experiments done by Akiu et al. (1991) with B16 mouse melanoma cells in culture showed that the hydroquinone moiety may not be responsible for arbutin's action. Although hydroquinone is up to 80% effective in the treatment of cutaneous hyperpigmentary disorders (Arndt and Fitzpatrick, 1965), arbutin has the advantage of being safer.

Considering the importance of arbutin for its therapeutic use against melasma, freckles, senile lentigines, etc., we have further investigated its effects on tyrosinase related proteins in the melanin synthetic pathway to clarify the mechanism of its depigmenting action in human melanocytes in culture. Such studies may enlighten the possibility of enhancing arbutin's effect on hyperpigmentary disorders.

MATERIALS AND METHODS
Cell Culture and Treatment

Normal human melanocytes were established in culture from foreskins of 18- to 40-year-old Japanese males. Melanocytes were maintained in Ham's F10 medium supplemented with 5% fetal calf serum, 85 nM TPA, 0.1 mM IBMX, 1.0 mM insulin, and 40 μg protein/ml bovine pituitary extract (Funasaka et al., 1992). During treatment with

arbutin, melanocytes were maintained in keratinocyte serum free medium (Keratinocyte SFM, GIBCO Lab., Life Technologies, Inc., NY) supplemented with 2 ng/ml basic fibroblast growth factor (bFGF, Sigma Chemical Co., St. Louis, MO). Human melanoma cells were cultured with Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum as usual, and all culture media contained appropriate amounts of antibiotics, penicillin and streptomycin.

A stock solution of arbutin (Nippon Fine Chemical Co., Ltd., Osaka, Japan) was made at the concentration of 10 mg/ml in PBS without Ca^{++} and Mg^{++}, and was added to the cell culture at final concentrations of 30, 100, and 300 µg/ml every day, every other day, or every other 2 days for 3 and 5 days.

Cell Proliferation Determination

Cells were collected by minimal trypsin/EDTA treatment and counted with a Fuchs-Rosenthal cytometer.

Assay of Melanin Content

The colors of cell pellets were evaluated visually, and pellets of 10^6 cells was solubilized by boiling in 1.0 M NaOH for 10 min. Spectrophotometric analysis of melanin content was done at 400 nm absorbance, as described previously (Oka et al., 1996).

Tyrosinase Assay

Cell pellets were lysed in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The radiometric determination of tyrosinase activity was performed as previously described by Pawelek (1978). In brief, 0.09 ml of each cell extract (20 µg of protein content) was incubated with 0.01 ml sodium phosphate buffer (0.1 M, pH 6.8) containing 1 µCi of L-tyrosine [ring-3,5-^{3}H] (Amersham, Buckinghamshire, England; Specific Activity 52 Ci/mmol), 5 µg of L-DOPA, and 1% Triton X-100 for 60 min at 37°C. One ml of activated charcoal (10% w/v) in 0.1 M citric acid was then added and specimens were centrifuged for 10 min at 2,000g at 4°C. The supernatants were applied to 0.2 ml columns of Dowex-50 equilibrated in 0.1 M citric acid, washed with 0.5 ml of 0.1 M citric acid, and the effluents were counted by scintillation spectrometry for the formation of ^3H_2O.

DOPAchrome Tautomerase (DT) Assay

Spectrophotometric assay of DT activity was carried out using DOPAchrome as substrate (Korner and Pawelek, 1980). DOPAchrome was synthesized by mixing ice-cold L-DOPA (0.75 mg/ml) in sodium phosphate buffer (0.1 M, pH 6.8) with solid Ag_2O (30 mg Ag_2O: 1 mg L-DOPA) for about 1 min and then filtering the mixture twice through Gelman Acrodisc Disposable Filters (number 4192, 0.2 µm diameter pore). The standard assay consisted of 0.1 ml DOPAchrome solution and 0.1 mg protein cell extract in a total volume of 0.2 ml sodium phosphate buffer (0.1 M, pH 6.8). The disappearance of absorption at 475 nm was noted with time. Phenylthiourea (0.1 mM) was included in DT assays

to inhibit endogenous tyrosinase, which can interfere with the assay.

DHICA Polymerase Assay

The assay was done according to Chakraborty et al. (1996) as described previously. The cell extract (0.5 ml, 200 µg of protein) was passed through a wheat germ agglutinin column (1 ml bed volume) equilibrated with lysis buffer. The eluate was recycled through the column 5–6 times. The column was washed once with NaCl (0.4 M, 0.5 ml) and two times with lysis buffer (0.5 ml). Bound material was eluted by washing twice with N-acetyl glucosamine (0.25 ml, 1 M, in lysis buffer) and the eluates were pooled (total vol. 0.5 ml). The glycoprotein-enriched eluate, which contains crude DHICA polymerization factor and other melanogenic proteins, was either divided into aliquots and stored frozen or was further concentrated with a Centriprep Concentrator. Owing to the sensitivity of DHICA to oxidants, all purification procedures were carried out in parallel with a lysis buffer blank that was then used as a control for background auto-oxidation in the assay.

DHICA, obtained from John Pawelek (Yale University School of Medicine, New Haven), was 97% pure by HPLC analysis (Chakraborty et al., 1996). DHICA was dissolved immediately prior to use in assays in 100 mM sodium phosphate (pH 6.8), at a stock concentration of 1 mg/ml (5 mM) by mixing at top speed on a Vortex Genie mixer for 1–2 min then filtering through a 0.22 µm diameter filter (Gelman Acrodisc). The light purple color supernatant solution was stored on ice before use.

A reaction mixture containing either the enzyme preparation to be measured (20 µg protein from wheat germ agglutinin eluates) or the appropriate buffer blank, and DHICA (0.5 mM), was adjusted to 0.2 ml with 100 mM sodium phosphate, pH 7.0, in a plastic multiwell plate. Phenylthiourea (0.1 mM) was also included to inhibit endogenous tyrosinase activity in the preparation. The reaction mixtures with appropriate controls were incubated at 37°C and the absorbance was recorded with a spectrophotometer at 400 nm for periods up to 4 h. DHICA-melanin, but not DHICA itself, has been shown to absorb light at these wavelengths (Orlow et al., 1992). An increase in absorbance over that seen in blank tubes was defined as specific DHICA polymerization factor activity (Chakraborty et al. 1996).

Protein Determinations

Protein content was measured using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

Western Blotting

Proteins from Nonidet P-40/SDS (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, 100 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) solubilized cells were separated on 7.5% SDS gels and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). The membranes were blocked with 5% BSA and incubated with

specific peptide antisera (1:1,000 dilution). The following antisera were used for these experiments. Polyclonal anti-PEP7 and anti-PEP8 antibodies, which recognize the COOH termini of the albino-protein (tyrosinase) and of the slaty protein (TRP-2), respectively, were obtained from Dr. V. J. Hearing, National Institutes of Health, Maryland (Jimenez et al., 1991; Tsukamoto et al., 1992), and an anti-human TRP-1 monoclonal antibody, TA-99, was obtained from Dr. S. Vijaysaradhi, Bowman-Gray School of Medicine, North Carolina (Vijaysaradhi et al., 1990). Normal rabbit serum was used as control. Subsequent visualization of antibody binding was carried out with Enhanced Chemiluminescence (Amersham Corporation) according to manufacturer's instructions.

Statistical Analysis

Statistical analyses of data pooled from three independent experiments were performed using the two-tail paired t-test with the help of STATVIEW program (Abacus Concepts, Inc., Berkeley, CA). The differences were considered significant if $P < 0.05$.

RESULTS
Cell Proliferation (Fig. 1A,B)

Different concentrations of arbutin were tested on the growth of human melanoma cells and also on normal human melanocytes. Both types of cells grew well even in the presence of 100 μg/ml arbutin for 5 days. However, 300 μg/ml of arbutin showed cell toxicity and cells detached from the dishes within 48 h (data not shown).

Melanin Formation (Fig. 2)

Arbutin treatment at 100 μg/ml concentration for 5 days showed a significant inhibitory effect (approximately 20%) on melanin formation by human melanoma cells (Fig. 2B). A representative pellet color is shown in Figure 2A. A shorter time period of treatment, 2–3 days, was also tested with arbutin (100 μg/ml) and some inhibition of melanin formation (approx 7%) was found (data not shown). Similar results were obtained with normal human melanocytes (data not shown).

Tyrosinase, DT, and DHICA Polymerase Activities (Figs. 3, 4)

Assay of melanogenic proteins before and after arbutin treatment revealed that inhibition of melanin formation correlated with inhibition of tyrosinase and DHICA polymerase activities. Significant inhibition, about 40–50% ($P<0.01$), of both tyrosinase and DHICA polymerase activities were observed after treatment of human melanoma cells with arbutin at 100 μg/ml for 5 days. At 30 μg/ml arbutin, a slight inhibition was also noticed in tyrosinase activity (Fig. 3).

Tyrosinase activity of normal human melanocytes was inhibited to a higher degree; 40–50% ($P<0.05$) and 70–80% ($P<0.01$) at 30 μg/ml and 100 μg/ml of arbutin, respectively (Fig. 4). However, DHICA polymerase activity of these samples was not tested due to the paucity of the material.
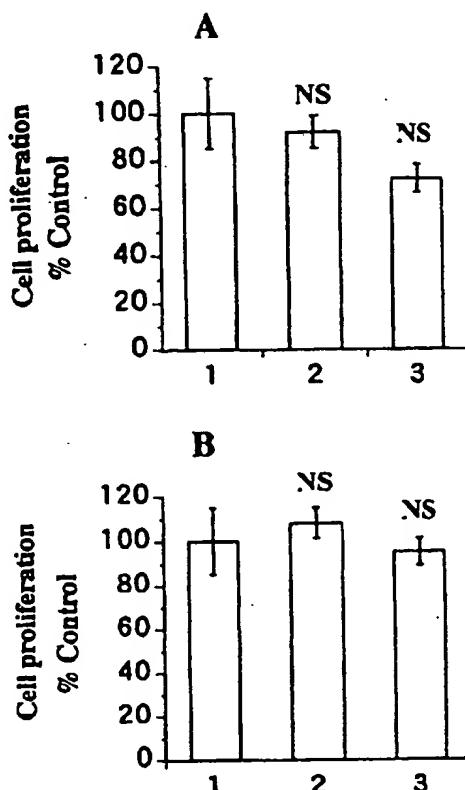
Fig. 1. Cells were counted with a Fuchs-Rosenthal cytometer after 5 days of treatment of human melanoma cells (A) or normal human melanocytes (B) with or without arbutin, as described in Materials and Methods. 1, Control; 2, 30 μg/ml arbutin; 3, 100 μg/ml arbutin. Results are expressed as % of control and data reported are means ± SD of at least three determinations.

Arbutin had no effect on DT of either type of cell at any dose or time point tested in these experiments (Figs. 3, 4).

Western Blotting (Figs. 5, 6)

Western blotting experiments demonstrated that there were no changes in the quality or quantity of tyrosinase, DT (TRP-2), or TRP-1 from human melanoma cells (Fig. 5) and/or human normal melanocytes (Fig. 6) after treatment with 100 μg/ml arbutin for 5 days.

DISCUSSION

Arbutin inhibits melanin synthesis significantly within 5 days of treatment of human melanoma cells with a dose of 100 μg/ml (Fig. 2). This inhibition was found to correlate with the inhibition of tyrosinase and DHICA polymerase activities but without any effect on cell growth (Fig. 1). Preliminary experiments with different doses of arbutin also showed concentration and time dependent inhibition of tyrosinase and DHICA polymerase activities in normal human melanocytes in culture (data not shown). However, no
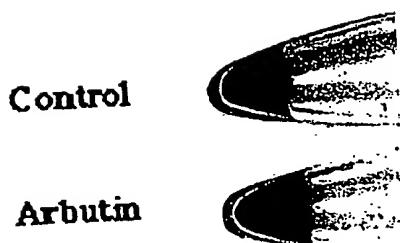
Fig. 2. Melanin content of human melanoma cells after 5 days of treatment with or without arbutin is demonstrated from a representative experiment. A: Photograph of cell pellets. B: Melanin content assay was done as described in Materials and Methods. Results are expressed as % of control and data are means ± SD of at least three determinations.
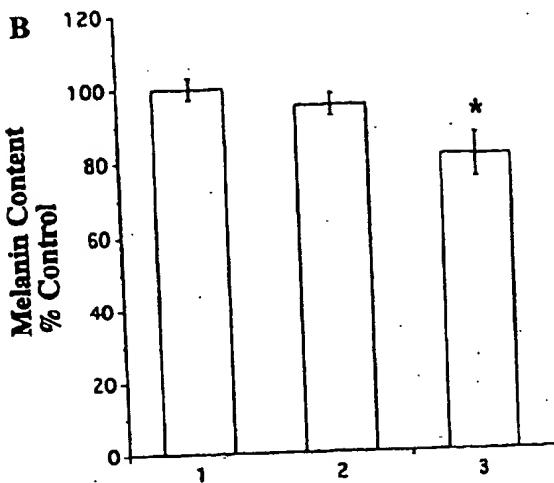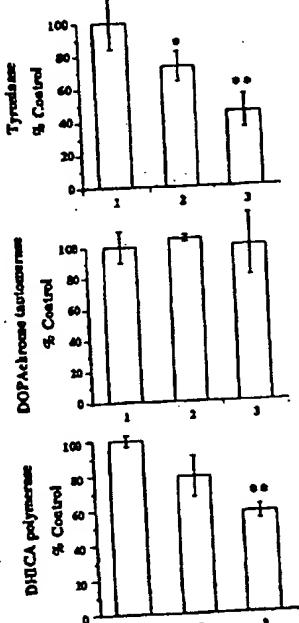
Fig. 3. Tyrosinase, DOPAchrome tautomerase (DT) and DHICA polymerase activities were measured in human melanoma cells after treatment with or without arbutin for 5 days as described in Materials and Methods. 1,Control; 2, 30 µg/ml arbutin; 3,100 µg/ml arbutin. Results are expressed as % of control and data are means ± SD of at least three determinations. *P<0.05; **P<0.01.
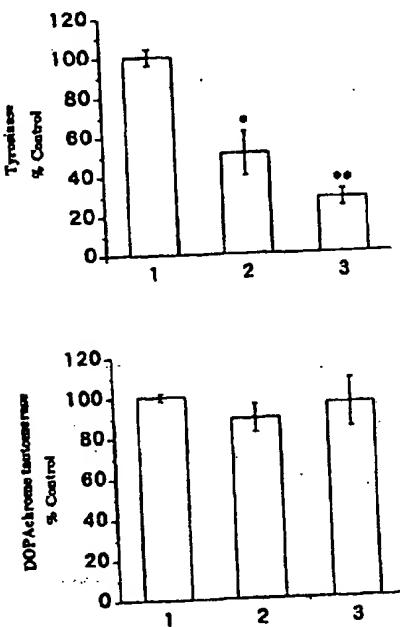
Fig. 4. Tyrosinase and DOPAchrome tautomerase (DT) were measured in normal human melanocytes after treatment with or without arbutin for 5 days as described in Materials and Methods. 1, Control; 2, 30 µg/ml arbutin; 3, 100 µg/ml arbutin. Results are expressed as % of control and data are means ± SD of at least three determinations. *P<0.05; **P<0.01.
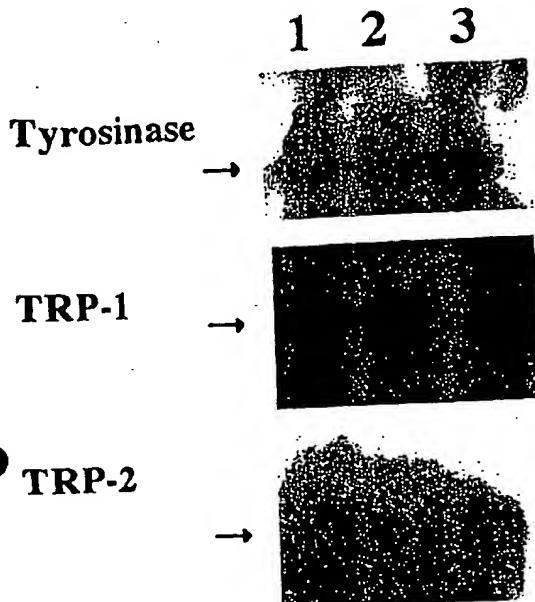
Fig. 5. Western blotting of tyrosinase, TRP-1 and TRP-2 in human melanoma cells with or without arbutin treatment for 5 days as described in Materials and Methods. 1, Control; 2, 30 μg/ml arbutin; 3, 100 μg/ml arbutin. Arrows indicate the representative bands, and no significant changes were noted. Similar results were obtained with repeated experiments.

Fig. 6. Western blotting of tyrosinase, TRP-1 and TRP-2 in normal human melanocytes with or without arbutin treatment for 5 days as described in Materials and Methods. 1, Control; 2, 30 μg/ml arbutin; 3, 100 μg/ml arbutin. Arrows indicate the representative bands, and no significant changes were noted. Similar results were obtained with repeated experiments.

significant changes were observed for DT activity from either type of cells before or after treatment with arbutin. It should be mentioned that human melanocytes express lower quantities of TRP-2 (DT) than do murine melanoma cells (Martinez-Esparza et al., 1997). Furthermore, in human melanoma cells it has been reported that there is no clear indication of a functional relationship between DOPAchrome tautomerase activity and the level of pigmentation (Bouchard et al., 1994).

We did not attempt to measure the TRP-1 activity in this present experiment, although we believe that TRP-1 is a predominant and related melanogenic protein (Vijaysaradhi et al., 1991; Jackson 1988; Jackson et al., 1990) but with inconclusive function. This molecule has been hypothesized to have differing enzymatic functions, including DHICA oxidase (Jimenez-Cervantes et al., 1994; Kobayashi et al., 1994), tyrosine hydroxylase (Jimenez et al., 1991, 1994; Zhao et al., 1994), DOPA oxidase (Jimenez et al., 1991, 1994), DHI oxidase (Hearing, 1993), DOPAchrome tautomerase (Winder et al., 1993), and catalase (Halaban and Moellmann, 1990). It has also been documented that TRP-1 is a key component of a melanogenic complex (Orlow et al., 1994), which functions to stabilize tyrosinase activity in vitro (Hearing et al., 1992; Winder et al., 1994; Zhao et al., 1996).

Western blotting experiments did not reveal any changes either in protein content or molecular size of tyrosinase,

TRP-2, or TRP-1 (immunoblotting for Pmel17/silver locus protein as DHICA polymerase was not studied). In contrast to murine melanoma cells, no correlation between tyrosinase activity and mRNA in human melanocytes and melanoma cells has been reported by several laboratories (Hoganson et al., 1989; Fuller et al., 1990; Naeyaert et al., 1991). Therefore, it appears from our results that the inhibition of tyrosinase by arbutin may be at the post-translational level.

Tyrosinase converts tyrosine to DOPAchrome which is converted to DHICA by DT, and DHICA polymerase catalyzes the polymerization reaction of DHICA to melanin. We measured the effects of arbutin on the activities of these melanogenic proteins and found that like tyrosinase, DHICA polymerase activity is also inhibited by arbutin. A similar inhibitory effect of arbutin on tyrosinase was also noted by others in human melanocytes in vitro (Maeda and Fukuda 1996). They also found decreased production of melanin intermediates, DHICA (the product of DT), after arbutin treatment. Since DHICA is a melanogenic precursor, its intracellular level could be a directive of how efficiently melanin formation can occur in vivo. The intracellular level of DHICA depends not only on DT activity but also on availability of its substrate, DOPAchrome, as well. Furthermore, it was reported before that in amphibian skin and in melanoma cells, indoleamine 2,3-dioxygenase (IOD) can break down indole molecules including DHICA and DHI, and hydroquinone stimulates that IOD activity

(Taylor and Feng, 1991; Chakraborty et al., 1993). Since we did not find any inhibition of DT by arbutin, the decreased level of DHICA observed by Maeda and Fukuda (1996) after arbutin treatment in human melanocytes in culture could be explained by decreased availability of DOPA-chrome due to inhibition of tyrosinase by arbutin, although an increased breakdown of DHICA, still remains as one possible cause.

Recently, Nakajima et al. (1998) reported the increase in pigmentation of cultured human melanocytes after arbutin treatment, although they found inhibition of tyrosinase activity, and suggested a mechanism other than tyrosinase that arbutin augments melanogenesis. They discussed various possibilities, which may explain the observed increased melanogenesis by arbutin despite its inhibitory effect on tyrosinase. Without repeating those we can mention that their observations were with a much higher concentration of arbutin, 2–8 mM (0.5–2 mg/ml), whereas our experiment was designed with a dose of arbutin that was not toxic to the cells, and we found that 100 µg/ml of arbutin is the best and safest concentration, and higher levels cause cell toxicity and detachment from dishes within 48 h. At a comparable dose, 0.5 mM (136 µg/ml), to our working concentration, 100 µg/ml, Nakajima et al. (1998) found no significant cell toxicity nor increase in pigmentation but a decrease in tyrosinase activity. Furthermore, since their treatment with arbutin was rather short, 3 days, we refrain from any further comparison with our observations.

Since melanin synthesis is also dependent on the transport of tyrosine, the precursor of melanin, into melanocytes, and arbutin inhibits induction of melanin formation by L-tyrosine in Bomirski hamster melanoma cells (Bolognia et al., 1991), it is possible that arbutin may also inhibit tyrosine uptake by cells, as well as compete with the substrate, tyrosine, at the active site of tyrosinase (Maeda and Fukuda, 1996). However, only slight inhibition of tyrosinase by arbutin was reported when it was added to a cell free extract of mushroom or B16 melanoma cells (Maeda and Fukuda, 1996). At comparable concentration hydroquinone is more effective than arbutin in causing tyrosinase inhibition, as was observed in our preliminary experiment (data not shown) and by others (Maeda and Fukuda, 1996). But arbutin is less cytotoxic than hydroquinone and thus has the advantage of safety.

In this study we found that arbutin was effective in inhibiting melanin formation in viable melanocytes and that this correlated with the inhibition of tyrosinase and DHICA polymerase activities. Since tyrosinase and pmel17/silver protein (DHICA polymerase) activities are both associated with the maturation of melanosomes; it may be possible that arbutin has an inhibitory effect on the maturation of melanosomes. Future studies in this direction are underway and will be presented in the next issuing paper.

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The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing

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ABSTRACT

We have determined the complete primary structure (8031 base pairs) of an infectious clone of cauliflower mosaic virus strain CM1841. The sequence was obtained using the strategy of cloning shotgun restriction fragments in the sequencing vector M13mp7. Comparison of the CM1841 sequence with that published for another CaMV strain (Strasbourg) reveals 4.4% changes, mostly nucleotide substitutions with a few small insertions and deletions. The six open reading frames in the sequence of the Strasbourg isolate are also present in CM1841.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the type member of the caulimovirus group, plant viruses containing circular, double-stranded DNA genomes (1). Since the great majority of plant viruses contain RNA genomes, CaMV has received considerable attention as a possible model for studying gene expression in plants (2) and as a potential vector for introducing foreign genetic material into plants (3).

A variety of CaMV strains have been isolated that differ in both phenotype and nucleotide sequence, as determined by restriction endonuclease analysis (4). However, further genetic analysis has proceeded slowly due to the difficulty in identifying viral gene products by conventional means. For example, several in vivo transcription products have been isolated, all of which are transcribed from only one of the DNA strands, referred to as the α-strand (5-7). A gene product has been identified for only one of these transcripts. A 61-66 kilodalton protein found in cytoplasmic inclusion bodies has been shown to be encoded by EcoRI fragments B and D of strain CM4-184 on the basis of hybridization and in vitro translation studies (7-9). Recently we have identified the region of the genome that expresses viral coat protein antigen in Escherichia coli (10).

With the advent of DNA sequencing technology direct information about the primary structure of DNA can be rapidly obtained (11, 12). From the sequence, information about genomic organization can be deduced. We report here the complete nucleotide sequence of an infectious clone of CaMV strain CM1841 obtained using a novel sequencing strategy described by Messing et al. (13). During the course of this work Franck et al. (14) published the sequence of the Strasbourg strain of CaMV, which they determined for DNA extracted from virus particles and sequenced by the chemical cleavage method of Maxam and Gilbert (11). Their sequence showed the presence of six open coding regions closely spaced in the genome with no evidence for intervening sequences.

We elected to sequence an infectious clone rather than a preparation of viral DNA for two reasons. First, restriction endonuclease cleavage patterns of viral DNA extracted from infected plants exhibit a range of minor fragments, some of which may be attributable to variability in sequence among the population of molecules (15-17). Secondly, CaMV viral DNA contains three single strand interruptions with an unusual structure (18, 14) which may cause difficulties during cloning (19). Both these problems are overcome by sequencing an infectious molecule that has been amplified in E. coli.

The sequencing strategy we employed involves shotgun cloning in the phage vector M13mp7 which contains cloning sites for fragments generated by a wide range of restriction endonucleases (13). This system makes it possible to determine the complete primary structure of large DNA's using standard cloning techniques. Strategies to optimize this method of sequencing are discussed herein along with the presentation of the sequence and genetic analysis.

MATERIALS AND METHODS

Bacteria and Plasmids

Plasmid pCaMV10 contains a full-length copy of CaMV strain CM1841 cloned at the SalI site of pBR322 (see 20). The clone is infectious, since typical mosaic symptoms develop when turnip leaves are inoculated with 0.1-0.5 µg of SalI-cleaved pCaMV10 DNA. Two preparations of pCaMV10 DNA were used as a source of starting material for cloning and sequencing, one grown in E. coli strain C600 (thi⁻ thr⁻ lac⁻ supE44, ref. 21), and one in strain GM48 (dam-3, dcm-6, thr-1, leu-6, thi-6, lacY, galK2, galT22, ara-14, tonA-3, tsx78, supE44, ref. 22). Both of the preparations were shown to be

capable of infecting turnip plants.

M13mp7 is a phage vector containing cloning sites for EcoRI, BamHI, SalI, AccI, HincII, PstI (described in ref. 13). M13mp2 (23), M13mp5 (a HindIII vector, ref. 24), and JM103, the E. coli host used to propagate M13 (13), have all been previously described.

Cloning

Restriction enzymes EcoRI, BamHI, SalI, XhoI, AccI, TaqI, HpaII, HaeIII, HindIII, BclI, BglII, MboI, and AluI were purchased from New England Biolabs, HincII (lot 2651) was purchased from Bethesda Research Laboratories. Sau3A was a gift of R. Gelinas. All restriction enzymes were used as recommended in the New England Biolabs catalogue. EcoRI* cleavage was performed in 20 mM Tris (pH 8.8), 2mM $MgCl_2$, 20% glycerol, for 16 h at 37°C using -10 units of enzyme per µg DNA.

Prior to restriction cleavage and cloning in M13, the CaMV moiety of pCAMV10 was purified away from the pBR322 vector by sucrose gradient centrifugation for 16 h in an SW 41 Ti rotor, and extraction of the faster moving band, as described in Lane and Gardner (25). When specific subfragments of the CM1841 genome were required, DNA fragments were extracted from Sea Plaque agarose gels (as described in ref. 20).

For cloning, DNA of the M13 vector and CaMV were cleaved with the relevant restriction enzyme, extracted with phenol, and ethanol precipitated prior to ligation. Ligation was performed in 50 mM Tris pH 8.2, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, at 4°C for -16 hr. T4 DNA ligase was purified by the method of Tait et al. (26). For ligation of cohesive-ended fragments, we used 5-10 units of ligase per µg DNA; for blunt-ended fragments, we used 500-1000 units ligase per µg DNA, and incubated the mixture at room temperature.

We initially had problems with blunt-ended cloning in M13mp7, since phage DNA cleaved with HincII and ligated without added CaMV DNA gave a large (30-40%) background of colorless plagues. However, use of one particular batch of HincII (BRL lot 2651) alleviated this problem, and resulted in 80-90% of the colorless plagues containing an CaMV insert.

The size of an insert in M13 was assayed by adding SDS (0.2% final concentration) to 10 µl of culture supernatant, followed by electrophoresis on agarose gels. Inserts of 100 bp or larger can be detected from the mobility of the ssDNA. In addition, we developed a simple and rapid hybridization assay to identify clones. Ten µl from the culture supernatants of two different clones were combined, SDS added to 0.2%, and the

mixture incubated at 65° for 1 h. If the inserts were complementary
(contain the same region of DNA cloned in the opposite orientation) they
will hybridize to form a "figure eight" structure that shows a reduced
mobility during electrophoresis in agarose. Thus it is possible to easily
identify a clone in the opposite orientation from one that already existed,
by hybridizing the two DNAs and carrying out gel electrophoresis. Similarly,
it is feasible to screen a clone bank for fragments from any region of the
genome. The hybridizing inserts need not be of equal size. For example we
have used a probe with a 3500 bp CaMV insert to identify clones of 200
nucleotides. Purified template DNA can also be used in this assay (see
ref. 20).

DNA sequencing

Growth of M13, purification of the ssDNA templates, and the dideoxy
chain termination reaction conditions used in these experiments are as
described in Howarth et al. (20). DNA polymerase I Klenow fragment was
purchased from New England Biolabs, New England Nuclear, or Bethesda Research
Laboratories. The 15 bp synthetic primer described by Messing et al. (13) was
used in all the sequencing reactions.

Computer analysis of the sequence was performed using the programs
of Korn et al. (27).

RESULTS AND DISCUSSION

Sequencing strategy

The restriction map of CM1841 has been established for several enzymes
(4, 20). Initially we cloned fragments of pCaMV10 in M13 using some of these
enzymes (EcoRI, BamHI, SalI, XhoI, BglII, and HindIII), and mapped the
inserts using restriction analysis. The DNA sequences obtained from
these clones could then be located on the pCaMV10 restriction map. This
initial step also provided us with a series of clones covering the whole
genome which we could use as hybridization probes or as sources for sub-
clones in subsequent steps.

We next cloned fragments generated by additional restriction enzymes
(MboI, EcoRI*, TaqI, HpaII, HaeIII, AluI, and HincII) whose cleavage sites
on the CaMV genome were not known. We determined the sequence of clones
chosen at random from the template bank. These clones were located on the
CM1841 genome by comparison with the sequence already determined, or by
comparison with the sequence of the Strasbourg strain of CaMV which had been
published in the meantime (14). From these data, we were able to locate

regions of the genome which had not been sequenced in one or both orienta-
tions. Clones covering these regions were obtained in one of three ways.
(a) Inserts of existing subclones were recloned in the opposite orientation.
The symmetry of the cloning sites in M13mp7 allowed this step for all
except EcoRI* clones. (b) Subclones were made from particular regions of
the genome using a suitable restriction endonuclease. For this purpose,
fragments from pCaMV10 or from a suitable subclone were purified from
agarose gels (see Materials and Methods). (c) To obtain the final se-
quences that were lacking, we searched through an existing bank of uni-
dentified AluI clones, using the hybridization assay to locate the required
insert (see Materials and Methods).

Randomness of the shotgun cloning procedure

The complete nucleotide sequence of the CM1841 part of pCaMV10 has been
determined from the sequence of over 175 different subclones (see Fig. 1).
Sequence information has been obtained on both strands for over 88% of the
genome. All of the sequence has been established from at least two indepen-
dent clones and 84% from three or more clones for each portion of the genome.
The entire sequence of 8031 nucleotides is shown in Fig. 2.

Table 1 shows the number of different clones generated by each restric-
tion endonuclease. In some cases, clones were screened for size before being
selected for sequencing, in order to obtain the particular clones which were
required. However, for banks generated by five different restriction enzymes,
selection of clones for sequencing was completely random. For four of these
enzymes (EcoRI* is discussed below), we tested whether the distribution of
clones fitted a Poisson distribution. In the case of MboI and AluI the re-
sults differed significantly ($p < 0.01$), suggesting that one MboI and one AluI
fragment of CaMV were cloned preferentially. It is of interest that both of
these fragments came from the same region of the genome. The MboI fragment
(2333→2149) was cloned seven times, always in one orientation. The AluI frag-
ment (1908→1678) was cloned three times in one orientation. In addition we
observed that repeated attempts to clone the small BamHI fragment from this
part of the genome always resulted in clones with the fragment in the same
orientation (2148→1926).

We are unable to suggest a persuasive argument for the cloning frequency
or the orientation of these fragments. It is unlikely that the clones ob-
tained are siblings, since the transformation procedure involves plating the
cells in soft agar immediately following the heat shock, i.e. there is no
growth period. The region of the CaMV genome in question has been shown to
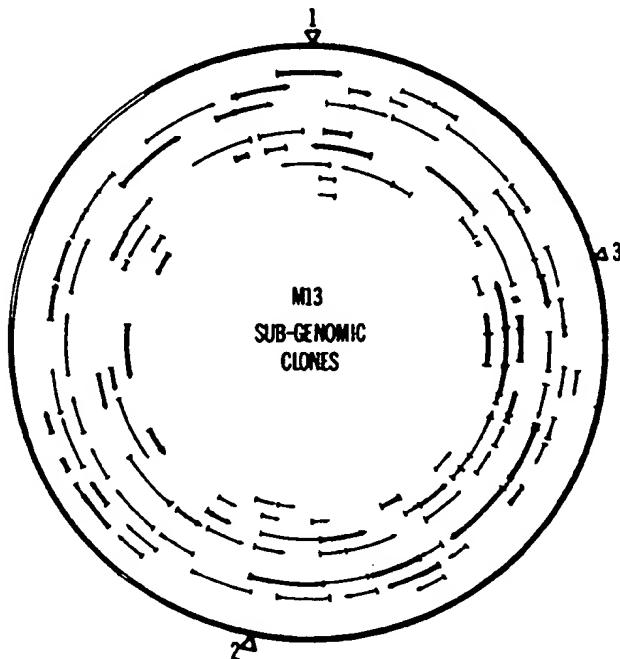
Fig. 1. Sequences used to construct the complete primary structure of
CM1841. The position of the three gaps in viral DNA are marked,
and the map is oriented with the origin at gap 1. Arrows show
the length and direction of the sequences obtained from subclones.
Thicker arrows indicate that a particular subclone was otained
twice or more. Arrows bounded at both ends by a stroke indicate
that both the start and finish of the inserted fragment were
identified in the sequence.

exhibit strong promoter activity in E. coli (28). The preferred orientation
would result in this promoter activity being in the same direction as the
transcription from the phage and lac promoters on M13mp7. However, we were
able to obtain CaMV subclones containing this region in the opposite orien-
tation using different restriction enzymes (HindIII, EcoRI*, HaeIII).

As shown in Table 1, the clone bank generated by EcoRI* contributed a
considerable portion of the complete nucleotide sequence. In addition it
has enabled us to identify 34 different EcoRI* cleavage sites on the CaMV
genome, and thus deduce the recognition specificity of sites cleaved by

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GTATCAGAG CCATGAATCG GTTTAAAAAC CAAACTCAAG ACGGTAAAAC CTCACCAAAA TACGAAAGAG TTCTTAACTC TAAAGATAAA   90
AGATCTTTCA ACATCAAAAC TAGTTCCCTC ACACCGGTCA CCCACAGGTT TACCACCGTA ACGTTTCAGA ACAACATCGA ATGCGTTTAC  180
GCCAACTTCG ACTCTCAGCT CAAGTCGTCG TACGATGGTA GATCTAAAAA CATCAAGAAT CTAAGCCTTA AAAATCTTAG ATGTTATGAA  270
GCCTTCCTCA GGAAGTACCT TCTGGAACAA TAAATCTCTC TGAGAATAGT ACTCTAACGA GTATCCACAG CAAAAATAAT CTTCTGTGTT  360
GAGATOGATT TGTATCCAGA AGAAAATACC CAAAGCGAGC AATCGCAGAA TTCTGAAAAT AATATGCAAA TATTTAAGTC ACAAAATTCG  450
GATGGATTCT CCTCCGATCT AATGATCTCA AACGATCAAT TAAAAAATAT CTCTAAGACC CAATTAACTT TGGAGAAAGA AAAGATATTT  540
AAAATGCCTA ACGTTTTATC TCAAGTTATG AAAAAAGCCGT TTAGCAGGAA AAACGAGATT CTTTACTGCG TCTCGACAAA ACAATTATCA  630
GTGGACATTC ACGATGCCAC ACGTAAGGTA TATCTCCCTT TAATCACTAG ACAGCAGATA AATAAAAGAC TTTCTAGCTT AAAACCTGAA  720
GTCAGAAAGA TCATGTCCAT GGTTCATCTT GCAGCGGTCA AAATATTGCT TAAAGCTCAA TTTCGAAATG GGATTGATAC CCCAATCAAA  810
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GAAGATCAAC TCATACTTCG GACTAAGCAA ACACCCTTCG GAGTCCAAAT CAAAAAACCC GTCAGTTTTT AATACTCCAA AAAACATTTT 1620
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TGCAATTCAA TCCTTAGATA ATAAGATTGA ACCACAGCCC TTAACTAAAG AAGAAGTTAA AGAGCTAAAA CAATCGATTA ACTCGATCAA 1800
ACAAGGATTA AACAATATTA TTGGCTGAAA TGGCTAATCT TAATCAAATC CAGAAAGAAG TCTCTCAAAT CCTCAGTCAC CAAAAATCCA 1890
TGAAATCGGA TATAAAGCCT ATCTTAGAAT TGCTAGGATC CCAAAATCCT ACTAAAGAAA GCTTAGAAGC CGTTCCAGCG AAAATCGTTA 1980
ATGACTTAAC CAAGCTCATC AATCGATTGTC CTTGTAACAA AGAGATATTA GAAGCCTTAG GCAATCAGCC TAAAGACCAA CTAATAGAAC 2070
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TCAACAAGCA GAGAATTTGG GTCTCCAGCC CGTTGAAGAA CCCTATGAAG GAGTTCAAGA AGTATTCATC TTAGAATACA AAGAAGAGGA 3600

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CCAATTCGAT CTACATCAAG GGAAGACTCT ACTTCAAAGG ATACAAGAAG ATAGAGCTTC ACTGTTTTGT AGACACGGGA GCAAGCTTAT 3780

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CCAACAAAGA GGAGTTACTT ACACTCATTG GAGGAAAGAA GATCTTTTCT TCCTTCGACT GTAAGTGCGG ATTCTGGCAA GTTCTACTTG 4680

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ACAGCAATGA CAAAGAGACA TTGGCGGTAA TAAATACTAT AAAGAAATTC AGTATTTATC TAACTCCTGT TCATTTTCTG ATTAGGACAG 5490

ATAATACTCA TTTCAAGAGT TTTGTTAACC TTAATTACAA AGGAGATTCA AAACTTGGAA GAAACATCAG ATGGCAAGCA TGGCTTAGCC 5580

ACTATTCGTT TGATGTTGAA CATATTAAAG GAACCGACAA CCCACTTTGCG GACTTCCTTT CAAGAGAATT CAATAAGGTT AATTCCTAAT 5670

TGAAATCCGA AGATAAGATT CCCACACACT TGTGGCTGAT ATCAAAAAGG CTACTACCTA TATAAACACA TCTCTGGAGA CTGAGAAAAT 5760

CAGACCTCCA AGCATGGAGA ACATAGAAAA ACTCCTCATG CAAGAGAAAA TACTAATGCT AGAGCTCGAT CTAGTAAGAG CAAAAATAAG 5850

CTTAGCAAGA GCTAACGGCT CTTCGCAACA AGGAGACCTC CCTCTCCACC GTGAAACACC GGTAAAAGAA GAAGCAGTTC ATTCTGCACT 5940

GGCCACTTTT ACGCCAACTC AAGTAAAGGC TATTCCAGAG CAAACGGCTC CTGGTAAAGA ATCAACAAAT CCGTTGATGG CTAGTATCTT 6030

GCCAAAAGAT ATGAACCCAG TTCAAACTGG GATAAGGCTT GCAGTGCCAG GGGACTTTTT ACGTCCTCAT CAGGGAATTC CAATCCCACA 6120

AAAATCTGAG CTTAGCAGCA TAGTTGCTCC TCTCAGAGCA GAATCGGGTA TTCACCACCC TCATATCAAC TACTACGTTG TGTATAACGG 6210

TCCACACGCC GGTATATACG ATGACTGGGG TTGTACAAAG GCGGCAACAA ACGGCGTTCC CGGAGTTGCA TACAAGAAGT TTGCCACTAT 6300

TACAGAGCCA AGAGCCAGCAG CTGACGCGTA CACAACAAGT CAGCAAACAG ACAGGTTGAA CTTCATCCCC AAAGGAGAAG CTCAACTCAA 6390

GCCCAAGACC TTTGCGAAGG CCTTAACCAG CCCACCCAAAG CAAAAAGCCC ACTGGCTCAC GCTACGGAACC AAAAGGCCCA GCAGTGATCC 6480
```

ACCCCAAAA GAGATCTCCT TTGCCCCGGA GATCACCATG GACGACTTTC TCTATCTCTA CGATCTAGGA AGAAAGTTCG ACGGACAAGG 6570

TGACGATACC ATGTTCACCA CCGATAATGA GAAGATTAGC CTCTTCAATT TCAGAAAGAA TGCTGACCCA CAGATGGTTA GAGAGGGCTA 6660

CGCGGCAGGT CTCATCAAGA CGATCTACCC GAGTAATAAT CTCCAGGAGA TCAAATACCT TCCCAAGAAG GTTAAAGATG CAGTCAAAAG 6750

ATTCAGCACT AACTGCATCA AGAACACAGA GAAAGATATA TTTCTCAAGA TCAGAAGTAC TATTCCAGTA TGGACGGATTC AAGGCTTGCT 6840

TCATAAACCA AGGCAAGTAA TAGAGATTGG AGTCTCTAAG AAAGTAGTTC CTACTGAATC AAAGGGCATG GAGTCAAAAA TTCAGATGGA 6930

GGATCTAACA GAACTGGGGG TGAAGACTGG CGAACAGTTC ATACAGAGTC TTTTACGGACT CAATGACAAG AAGAAAATCT TGGTCAACAT 7020

GGTGGAGGAC GACACTCTGG TCTACTCCAA GAATATCAAA GATACAGTCT CAGAAGACCA AAGGGCTATT GAGACTTTTC AACAAAGGGT 7110

AATATCGGGA AACCTCCTCG GATTCCATTG CCCAGCTATC TGTCACTTCA TCAAAAGGAG AGTAGAAAAG GAAGGTGGCA CCTACAAATG 7200

CCATCATTGC GATAAAGGAA AGGCTATGGT TCAAGATGCC TCTGCCCGACA GTGGTCCCAA AGATGGACCC CCACCCACGA GCAGCATCGT 7290

GGAAAAAGAA GACGTTCCAA CCACGTCTTC AAAGCAAGTG GATTGATGTG ATATCTCCAC TGACGTAAGG GATGACGCAC AATCCCACTA 7380

TCCTTCGCAA GACCCTTCCT CTATATAAGG AAGTTCATTT CATTTGGAGA GGACACGCTG AAATCACCAG TCTCTCTCTA CAAATCTATC 7470

TCTCTCTATT TTGTGCATAA TAATGTGTGA GTAGTTCCCA GATAAGGGAA TTAGGGTTCT TATAGGGTTT GGCTCACGTG TTGAGCATAT 7560

AACAAACCCT TAGTATGTAT TTGTATTTGT AAAATACTTC TATCAATAAA ATTTCTAATT CCTAAAACCA AAATCCAGTA CTAAAATCCA 7650

GATCACCTAA AGTCCCTATA GATCTTTGTC GTGAATATAA ACCAGACATG AGACGACTAA ACCTGCAGCC CACAGCGCCGT TCGAAGCTAG 7740

AAGTACCGCT TAGGCAGGAG GCCGTTAGGG AAAAGATGCT AAGCCAGGGT TGGTTACGTT GACTCCCCCG TAGGTTTGGT TTAAATATGA 7830

TAAAGTGGAC GGAAGGAAGG ACGAAGACAA GGAAGGATAA GGTTGCAGGC CCTGTGCAAG GTAAGAAGAT GGAAATTTGA TAGAGGTACG 7920

TTACTATACC TATACTATAC GCTAACGGAT GCTTGTATTT TACCCTATAC CCCCTAATAA CCCCTTATCG ATTTTAAGAA ATAATCCGCA 8010

TAAGCCCCCG CTTAAAAAAT T 3031

Fig. 2. The nucleotide sequence of CaMV strain CM1841. The complete
nucleotide sequence (8031 bp) of the CaMV moiety of pCaMV10 is
shown. The sequence is given in the positive direction (the
direction of transcription) beginning at gap 1 (as defined in
ref. 14). Differences between this sequence and that published
for Strasbourg strain (14) are marked above the line: *=nucleotide
substitution, +=nucleotide insertion, and Δ=nucleotide deletion, in
CM1841 relative to Strasbourg.

EcoRI* under the cleavage conditions used (28a). A proportion of the EcoRI*
cleavage sites do not contain a central AATT tetranucleotide. Fragments
bounded by such sites thus possess cohesive termini which are not completely
homologous to the EcoRI termini of M13mp2, and are obtained at considerably
lower frequency than expected. Thus the randomness of the EcoRI* clone
bank could not be evaluated.

Evaluation of the sequencing method

The distribution of subclones was sufficiently random that we were able
to obtain sequence data covering the entire CaMV molecule. However there
were two regions of the genome (nucleotides 750-1200, and 6950-7500) from

Table 1. Shotgun cloning from pCaMV10

Cloning enzyme	Total possible fragments	Clones sequenced	Number different	Repeats
EcoRI*	88a	47	31	5,4,3,3,2,2,2,2,2,
MboI	84	39	25	7,4,2,2,2,2,2,
TaqI	62	9	9	
AluI	82	12	9	3,2
HincII	12	3	3	
HindIII[b]	16	18	11	4,2,2,2,2
HaeIII[b]	26	17	8	3,3,3,2,2,2
HpaII[b]	24	10	7	2,2,2

[a]56 of these have one end "non-homologous" to the EcoRI AATT end of
M13mp2 (see text).

[b]Screened for size before sequencing.

    This table shows the results of the sequencing for some of the enzymes
used to make a bank of clones from pCAMV10. The total number of possible
fragments (second column) is obtained by doubling the number of restriction
sites in the genome (two sequences can be obtained from each site, one in
each direction) and subtracting two or four (the starting material was linear
at the SalI site, which eliminates two of the possible fragments for most
enzymes, or four in the case of TaqI and HincII). The number of different
sequences identified (column 4) among the total number of clones (column 3)
is shown for each enzyme. The fifth column shows the number of times we
obtained sequences that appeared more than once. For example, in the MboI
cloning, one sequence appeared 7 times, one four times, and five sequences
appeared twice. For the lower three enzymes, choice of clones for sequencing
was not entirely random since the inserts were screened for size before
sequencing. In the case of HaeIII and HpaII, only clones with large inserts
were sequenced. For HindIII a representative size distribution of inserts
was chosen for sequencing.

which relatively fewer clones were obtained, primarily because there are
fewer cleavage sites in these regions for the particular restriction enzymes
that we used. This difficulty is inherent in the method of generating tem-
plates for sequencing by shotgun cloning of restriction fragments. We over-
came this disadvantage by the use of the hybridization assay to identify
desired rare clones from the bank. An alternative approach would be the pre-

paration of fragments from the region in question and using these to prime
synthesis within a large M13 insert (see ref. 29).

Several modifications could be made to the sequencing strategy that we
used to improve the yield of information from each sequencing reaction. For
example: a) the DNA to be sequenced should be circularized by ligation before
subcloning, so that there is an equal chance of obtaining clones containing
all possible fragments; b) for restriction enzymes that cleave frequently, a
partial digest should be used for cloning to reduce the number of short in-
serts obtained; c) clone banks should be generated from as wide a range of
restriction enzymes as is feasible, and a small fraction of the total number
of possible clones from each bank should be chosen for sequencing. This ap-
proach would help to reduce the non-randomness that results from preferential
cloning of some restriction fragments, and from the particular distribution
of cleavage sites in the molecule for each enzyme.

Our results show the feasibility of sequencing a medium-sized molecule
using this approach. The method has several advantages. Sequence data can
be produced very rapidly. The technology involved in generating templates
for the sequencing reactions involves standard cloning methods. Once tem-
plates have been prepared, one person can readily obtain 1000 nucleotides
of sequence per day. More importantly, the method generates an enormous
resource: a bank of clones of widely differing sizes (in our case from 26
to 8031 bp) that covers the entire genome. These clones are strand specific,
and thus of particular use as hybridization probes and for subsequent
genetic manipulation.

Comparison of the sequences of CM1841 and Strasbourg

The nucleotide sequence we have determined for CM1841 is very similar
to that reported for the Strasbourg strain of CaMV (14). Differences between
the two sequences are primarily nucleotide substitutions, with only a few
small deletions and insertions (differences are marked in the sequence shown
in Fig. 2). Overall the changes represent 4.4 mutations per 100 nucleotides
(Table 2).

The six open reading frames found in the sequence of the coding α-strand
of Strasbourg are also present in CM1841. No frame shifts or stop codons
occur within any of the regions as a result of the differences in sequence.
In contrast, several of the insertions or deletions outside the coding re-
gions would cause frame shifts (see Table 2). Within each coding region, the
proportion of base changes that cause a corresponding amino acid change
(Table 2, last column) is significantly lower than expected on the basis of

Table 2. Comparison of nucleotide sequence between the CM1841 and Strasbourg strains of CaMV

Coding region	Map location (1st Met-stop)	Nucleotide changes			Amino acid changes		
		Substi-tutions	Other	%a	Number	%	per base change
I	364-1347	35	-	3.6%	7	2.1%	0.25
II	1349-1828	13	-	2.7%	6	3.8%	0.46
III	1830-2219	20	-	5.1%	5	3.9%	0.25
IV	2201-3667	82	Δ3,Δ3,+3	5.6%	19	3.9%	0.23
V	3633-5669	75	-	3.7%	8	1.2%	0.11
"Intergenic"	5670-5773	2	+1	2.9%	-	-	-
VI	5774-7336	88	-	5.6%	28	5.4%	0.32
"Intergenic"	7337-365	27	+9,Δ1,+1	2.9%	-	-	-
Total genome	0-8031	341	+14,Δ7	4.4%	73	3.2%	

aEach deletion or insertion counts as a single change in the %.

The differences between the nucleotide sequences of CM1841 and Strasbourg were examined for each of the six open reading frames and the two "intergenic" regions of the genome. For the purposes of the comparison with Strasbourg, Region V has been taken as starting from the second of two adjacent Met codons in CM1841. The nucleotide positions refer to CM1841 (Fig. 2), and the insertion or deletion of bases refers to CM1841 relative to Strasbourg (which contains 8024 nucleotides). The putative protein products of each open coding region were compared between strains, and the number and % of changes are shown in columns 6 and 7. The last column gives the proportion of nucleotide changes that cause amino acid changes for each region.

chance (with the exception of Region II, where the difference is not statis-
tically significant, p=0.2). Furthermore, many of the amino acid changes
that do occur between CM1841 and Strasbourg are conservative (see Fig. 3).
These observations provide circumstantial evidence that the reading frames
in the nucleotide sequence code for proteins. The large 'intergenic' region
in CM1841, like its counterpart in Strasbourg, contains stop codons in all
three frames and could accommodate only a small protein.

Differences in amino acid sequence between the putative protein products
of each of the six regions are shown in Fig. 3. Region V, the largest coding
region, shows easily the highest degree of conservation in protein sequence.
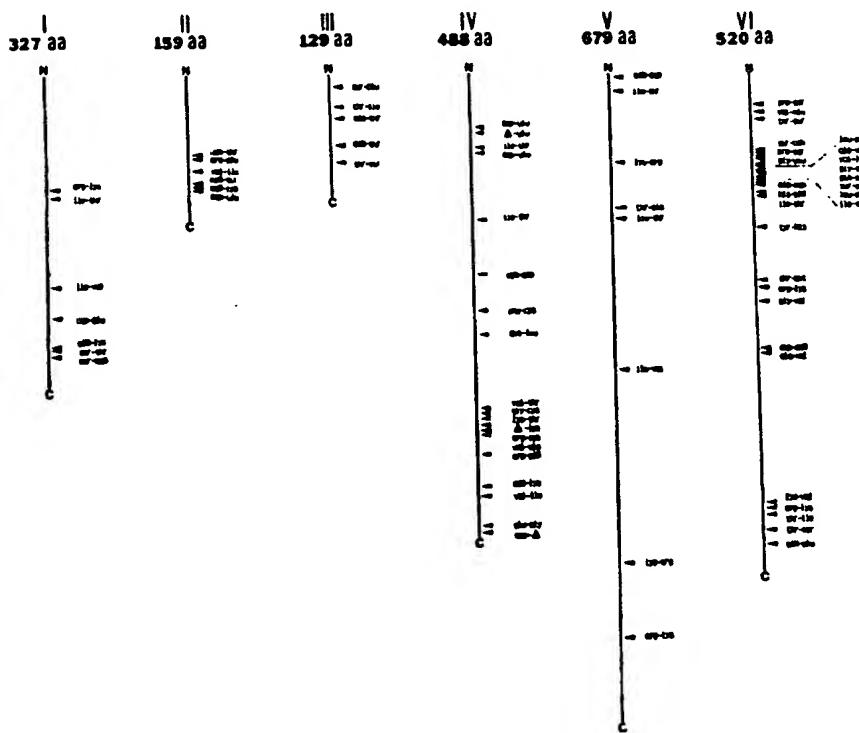
Fig. 3. Amino acid changes in the six coding regions. Amino acid residues
that differ between the putative protein products of CM1841 and
and Strasbourg are arrayed as a linear map for each of the six
coding regions on the genome. The CM1841 residue is given first
in each case.

The two regions for which gene products have been identified, Region IV and
Region VI (the coat protein and the inclusion body protein respectively,
see introduction), both show clusters of amino acid changes, some of which
involve potentially radical alterations.

The overall amino acid compositions of the six coding regions are unusual
in two respects. First, all six putative proteins have an unusually high
lysine content (molar ratios of 8.6, 11.9, 11.6, 11.3, 11.2, and 9.4%,
respectively). Secondly, both Region III and Region VI have a high proline
content (7.1 and 6.9%).

It may be somewhat premature to suggest a promoter function for various
CaMV sequences, since there is little biological data to support such identi-
fication. In addition, although promoter sequences have been identified for
some animal and animal virus genes (30-32), there is no corresponding data
from plants. With these reservations, we would like to point out two strate-
gically located examples of CaMV sequences that resemble promoter regions
found in animals. One such sequence is located before the first ATG of Region
VI, and contains both a "-70" region (nucleotides 5704-5712) and a "TATA" box
(5729-5736). Another promoter-like sequence occurs early in Region IV. The
"-70" region (2203-2211) and "TATA" box (2242-2246) could initiate trans-
cription just before the 2nd or 3rd ATG in Region IV.

The sequence data that we have obtained for CM1841 reveal a genetic
organization that is very similar to that for Strasbourg strain. This simi-
larity in organization parallels that observed previously for other DNA
viruses: SV40 and BK (33), fd and M13 (34), and φx174 and G4 (35). The de-
gree of difference between the DNA and protein sequences of these viruses is
shown in Table 3. The two strains of CaMV are not as closely related as the
filamentous bacteriophages fd and M13, but show much greater similarity than
the bacteriophages φx174 and G4, or the papovaviruses SV40 and BK.

The two CaMV strains exhibit some biological differences and have quite
distinct origins. CM1841 is a relatively mild strain of CaMV, isolated
in California in 1967 from Brassica spp. (see ref. 20). It is defective in
the ability to be transmitted by aphids, the natural vector for CaMV (36).
The Strasbourg strain (originally called the Milan isolate) was isolated
in Italy by G. Conti (see ref. 4). The strain is more severe than CM1841
(7), and is probably aphid transmissible. We know of no reports concerning
the serological relationship between CM1841 and the Strasbourg strain.

It is difficult to identify a genetic component for any of these biolo-
gical differences between the strains. The genetic basis of aphid trans-

Table 3. Sequence comparisons between related DNA viruses

Viruses	Genome Lengths	Size difference	% nucleotide changes	% amino acid changes
fd, M13	6408, 6407	1	3.0	0.6
Cabb-S, CM1841	8024, 8031	7	4.4	3.2
SV40, BK	5226, 4963	263	31.0	27.0
φx174, G4	5386, 5577	191	33.1	34.8

The differences between the sequences of DNA viruses that possess a similar genetic organization are shown. The data are taken from references 33-35, and this paper.

mission is unknown (see discussion of ref. 20). Recently we have shown that changes in Region VI can alter the severity of symptoms (37). Thus the cluster of changes in Region VI (Fig. 3) is an attractive candidate to account for differences in severity of the two strains. However symptom induction is likely to have a complex genetic basis, and to be affected by more than one region of the genome.

A large number of CaMV strains exist (4), and variants can be isolated at high frequency (38). We believe that impure viral cultures, resulting from a high multiplicity of infection during mechanical transmission, and perhaps by aphids in nature, has lead to the perpetuation of minor variants along with a major strain. We suggest that CaMV replication is a high fidelity process, and is not a factor in generating the observed variability. Two results from the sequence data support this assertion. We have shown recently that most of open reading region II has been deleted from CM4-184, a derivative of CM1841 (20). Therefore, Region II is clearly non-essential, at least for replication and cell-to-cell movement in plants in the green-house. We were suprised, therefore, to discover that the sequence of Region II in CM1841 did not diverge significantly from that of the Strasbourg strain (Table 2). We conclude that the Region II sequences in CM1841 have been faithfully replicated without selection since the isolation of that strain in 1967. Secondly, we have obtained over 600 bp of sequence infor-mation from strain CM4-184, the deletion variant derived from CM1841. The data is identical to the corresponding CM1841 sequence except for an A→T change at nucleotide position 7649. These two strains have been indepen-dently propagated since 1972.

Stable maintenance of non-essential DNA in the CaMV genome is a prerequisite to the use of this virus as a recombinant DNA vector in higher plants.

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Useful Proteins from Recombinant Bacteria

Bacteria into which nonbacterial genes have been introduced are able to manufacture nonbacterial proteins. Among the proteins made by recombinant-DNA methods are insulin and interferon

by Walter Gilbert and Lydia Villa-Komaroff

A living cell is a protein factory. It synthesizes the enzymes and other proteins that maintain its own integrity and physiological processes, and (in multicelled organisms) it often synthesizes and secretes other proteins that perform some specialized function contributing to the life of the organism as a whole. Different kinds of cells make different proteins, following instructions encoded in the DNA of their genes. Recent advances in molecular biology make it possible to alter those instructions in bacterial cells, thereby designing bacteria that can synthesize nonbacterial proteins. The bacteria are "recombinants." They contain, along with their own genes, part or all of a gene from a human cell or other animal cell. If the inserted gene is one for a protein with an important biomedical application, a culture of the recombinant bacteria, which can be grown easily and at low cost, will serve as an efficient factory for producing that protein.

Many laboratories in universities and in an emerging "applied genetics" industry are working to design bacteria able to synthesize such nonbacterial proteins. A growing tool kit of "genetic engineering" techniques makes it possible to isolate one of the million-odd genes of an animal cell, to fuse that gene with part of a bacterial gene and to insert the combination into bacteria. As those bacteria multiply they make millions of copies of their own genes and of the animal gene inserted among them. If the animal gene is fused to a bacterial gene in such a way that a bacterium can treat the gene as one of its own, the bacteria will produce the protein specified by the animal gene. New ways of rapidly and easily determining the exact sequence of the chemical groups that constitute a molecule of DNA make it possible to learn the detailed structure of such "cloned" genes. After the structure is known it can be manipulated to produce DNA structures that function more efficiently in the bacterial cell.

In this article we shall first describe some of these techniques in a general way and then tell how we and our colleagues Argiris Efstratiadis, Stephanie Broome, Peter Lomedico and Richard Tizard applied them in our laboratory at Harvard University to copy a rat gene that specifies the hormone insulin, to insert the gene into bacteria and to get the bacteria to manufacture a precursor of insulin. In an exciting application of this technology Charles Weissmann and his colleagues at the University of Zurich recently constructed bacteria that produce human interferon, a potentially useful antiviral protein.

DNA, RNA and Proteins

Cells make proteins by translating a set of commands arrayed along a strand of DNA. This hereditary information is held in the order of four chemical groups along the DNA: the bases adenine, thymine, guanine and cytosine. In sets of threes along DNA these bases specify which amino acids, the fundamental building blocks of proteins, are to be used in putting the protein together; the correspondence between specific base triplets and particular amino acids is called the genetic code. The part of a DNA molecule that incorporates the information to specify the structure of a protein is called a structural gene.

To act on this information the cell copies the sequence of bases from its genetic storehouse in DNA into another molecule: messenger RNA. A strand of DNA serves as a template for the assembly of a complementary strand of RNA according to base-pairing rules: adenine always pairs with uracil (which in RNA replaces DNA's thymine) and guanine pairs with cytosine. In animal cells transcription takes place in the nucleus of the cell. The messenger-RNA molecules carry the information out of the nucleus into the cytoplasm, where a complex molecular machine translates it into protein by linking together the appropri-

ate amino acids. In bacteria, which have no nucleus, transcription and translation take place concurrently. The messenger RNA serves as a temporary set of instructions. Which proteins the cell makes depends on which messengers it contains at any given time; to make a different protein the cell makes a new messenger from the appropriate structural gene. The DNA in each cell contains all the information required at any time by any cell of the organism, but each cell "expresses," or translates into protein, only a specific small portion of that information. How does the cell know which structural genes to express?

Along with the structural information, a DNA molecule carries a series of regulatory commands, also written out as a sequence of bases. The simplest of these commands say in effect "Start here" or "Stop here" both for the transcription and for the translation steps. More complicated commands say when and in which type of cell a specific gene should be used. The genetic code is the same in all cell nuclei, a given structural sequence specifying the same protein in every organism, but the special commands are not the same in bacteria and in animal cells. One of the most surprising differences was discovered only in the past two years. The information for a bacterial protein is carried on a contiguous stretch of DNA, but in more complicated organisms, such as pigs and people, the structural information is broken up into segments, which are separated along the gene by long stretches of other DNA called intervening DNA or "introns." In such a cell a long region (often 10 times more than might be needed) is transcribed into RNA. The cell then processes this long RNA molecule, removing the sequence of bases that does not code for the protein and splicing together the rest to make a messenger-RNA molecule that carries essentially just the "start," the structural sequence and the "stop" needed for translation.

74

To persuade a bacterium to make a nonbacterial protein one must put into bacteria a DNA molecule that has a sequence of bases specifying the protein's amino acids as well as the bacterial commands for transcription and translation. Moreover, the inserted DNA must be treated by the bacterium as its own so that it will be duplicated as the bacterium divides. The problem thus breaks down into three parts: to find the right structural sequence (insulin's, for example), to place it in bacteria in such a way that it will be maintained as the bacteria grow and then to manipulate the surrounding information, modifying the regulatory commands so that the structural sequence is expressed as protein. Once the protein is made, still further changes in its gene or modifications of the bacterium may be needed to obtain the protein in large enough amounts to be useful.

The constellation of recombinant-

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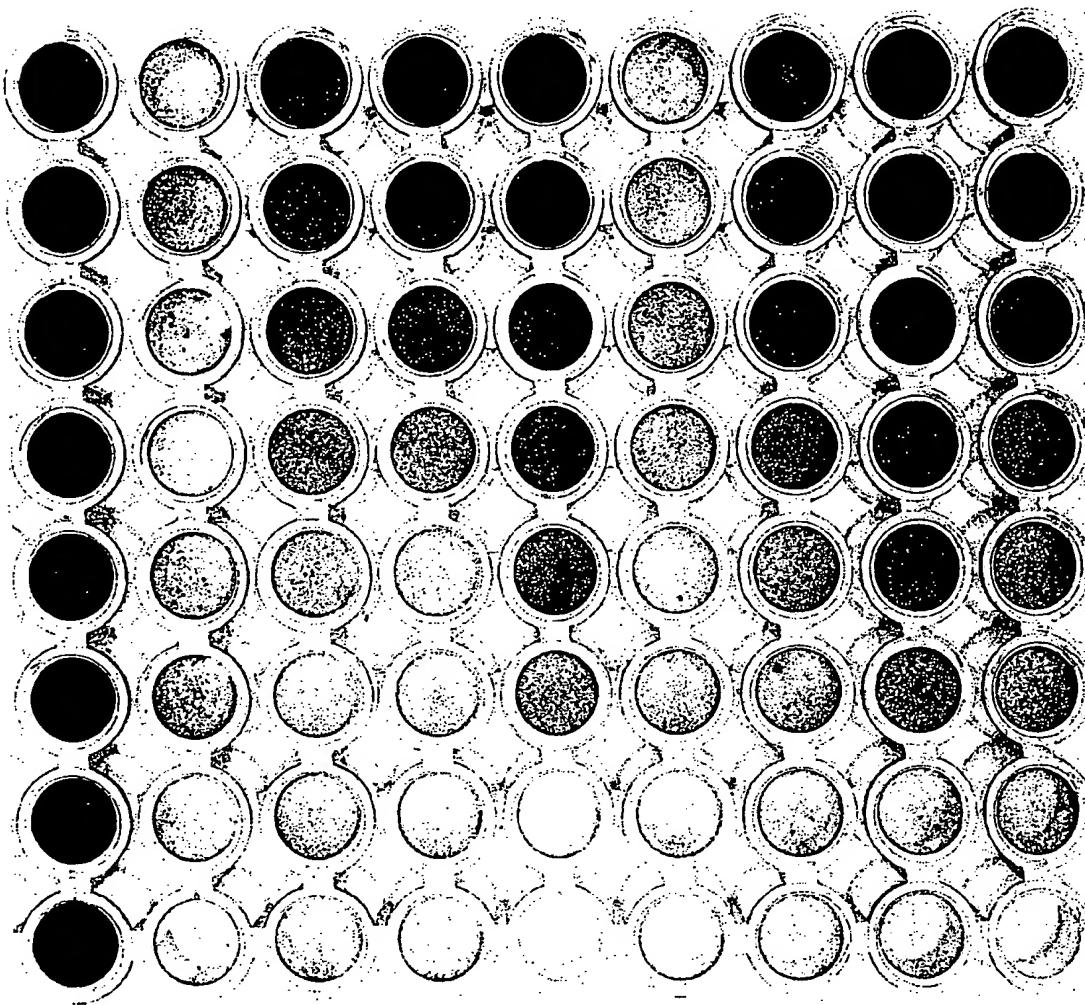
HUMAN INTERFERON synthesized in bacteria demonstrates its ability to block a viral infection in this biological assay. The structural information for making the protein interferon was obtained from human white blood cells in the form of messenger-RNA molecules; the RNA then served as a template for the synthesis of double-strand molecules of copy DNA, and the DNA in turn was inserted by recombinant-DNA techniques into a laboratory strain of the bacterium Escherichia coli, which synthesized the protein. For the assay dilutions of an extract of the bacteria were placed in some of the wells of a clear plastic tray; the other wells served as controls. (The wells are seen through the bottom of the tray in this photograph.) Human cells were added to the wells and were grown to form a layer of cells covering the bottom of each well. A virus preparation was then added to the cells. Twenty-four hours later the cell layer was stained. Where interferon in the extracts protected the cells against the virus the cells survived and were stained. Where there was no interferon the virus killed the cells and the dead cells did not pick up the stain. The control wells in the first column at the left contain a layer of cells that were never exposed to the virus; they accordingly appear stained. The control wells in the second column contain cells that have been killed by the virus; they look gray or clear. The control wells in the third column contain dilutions of a standard laboratory sample of interferon obtained directly from human cells; the top well has the most interferon and each succeeding well has a third as much interferon as the well above it. The wells in the next six columns hold dilutions of bacterial extracts from six different colonies of E. coli in which interferon DNA was present. Five of the six columns containing the bacterial extracts show evidence of interferon activity. The third extract tested (Column 6) had no detectable interferon; it apparently did not have a complete interferon gene. The synthesis of human interferon by the recombinant-DNA method was achieved by Charles Weissmann and his colleagues at the University of Zurich in collaboration with Kari Cantell of the Finnish Red Cross. The work was supported by Biogen, SA. Interferon is synthesized by many animal cells, but it is species-specific: only human interferon works for human beings, and it has been too scarce even for satisfactory experimentation.

75

DNA techniques for placing and maintaining a new gene in bacteria is called cloning, which in this sense means the isolation of a specific new DNA sequence in a single organism that proliferates to form a population of identical descendants: a clone. There are two convenient ways of doing this. In one method a small circular piece of DNA called a plasmid is the vehicle for introducing the new DNA into the bacterium. Plasmids carry only a few genes of their own and are maintained in several copies inside the bacterium by the bacterium's own gene functions; they remain separate from the main set of bacterial genes carried on a circle of DNA about 1,000 times larger. Alternatively the vehicle could be a virus that grows in bacteria. Such viruses normally have some 10 to 50 genes of their own (a bacterium has several thousand genes) and can often carry other new DNA segments in place of some of their own. All the techniques we shall describe apply to both plasmids and viruses.

A molecule of DNA resembles a very
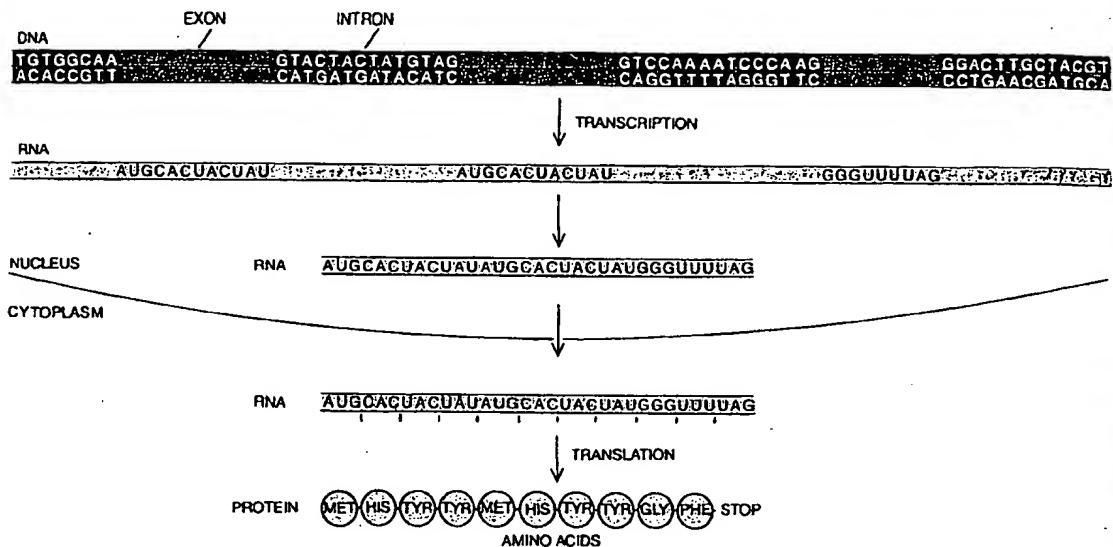
PROTEINS ARE MADE in a living cell according to instructions encoded in the cell's genes, which consist of specific sequences of chemical groups (bases) strung out along a double-strand molecule of DNA in the cell's nucleus. The genetic code is "written" in the four letters A, T, G and C, which stand respectively for the four bases adenine, thymine, guanine and cytosine. The code is "read" in the three-letter sets called codons, which specify the amino acids linked together in the protein chain. The order of the bases can also convey regulatory commands. In multicelled organisms the structural sequence, or gene, encoding a particular protein is usually broken into fragments separated by long stretches of other DNA; in this diagram the gene fragments, called exons, are represented by the black letters and the intervening sequences, known as introns, by the white letters. The genetic information is translated into protein indirectly. First the entire sequence of bases is transcribed inside the nucleus from the DNA to a single-strand molecule of RNA. According to the base-pairing rules governing transcription, adenine always pairs with uracil (U) and guanine always pairs with cytosine. Next the RNA copies of the introns are excised from the message and the remaining RNA copies of the exons are joined together end to end. The reassembled strand of messenger RNA then moves from the nucleus to the cytoplasm, where the actual protein-manufacturing process takes place.
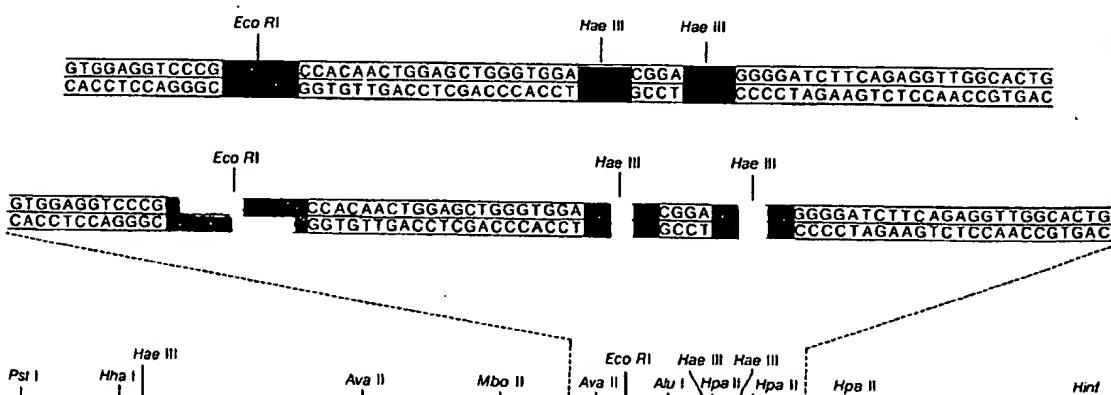
DNA CAN BE CUT into comparatively short lengths with the aid of restriction endonucleases, special enzymes that recognize specific base sequences at which they cause the molecule to come apart. For example, Eco RI, the first such enzyme discovered, recognizes a certain six-base sequence and cuts the molecule wherever this sequence appears, whereas Hae III, another restriction enzyme, operates at a certain four-base sequence. Since the probability of finding a particular four-base sequence is greater than that of finding a particular six-base sequence, one would expect Hae III to cut DNA more often than Eco RI. Accordingly one Eco RI site and two Hae III sites are represented in the DNA segment at the top, which corresponds to part of the gene coding for insulin in rat cells. The same DNA contains recognition sites for a number of other restriction enzymes, as is shown in the line diagram of a larger gene fragment at the bottom.

76

BACTERIAL PLASMID DNA

REVERSE
TRANSCRIPTASE

MESSENGER RNA

cDNA

ALKALI

cDNA

DNA POLYMERASE

cDNA

S1 NUCLEASE

DOUBLE-STRAND DNA

TERMINAL
TRANSFERASE

Pst

TERMINAL
TRANSFERASE

MIX AND
ANNEAL

UPTAKE BY CELL;
REPAIR BY
CELLULAR ENZYMES
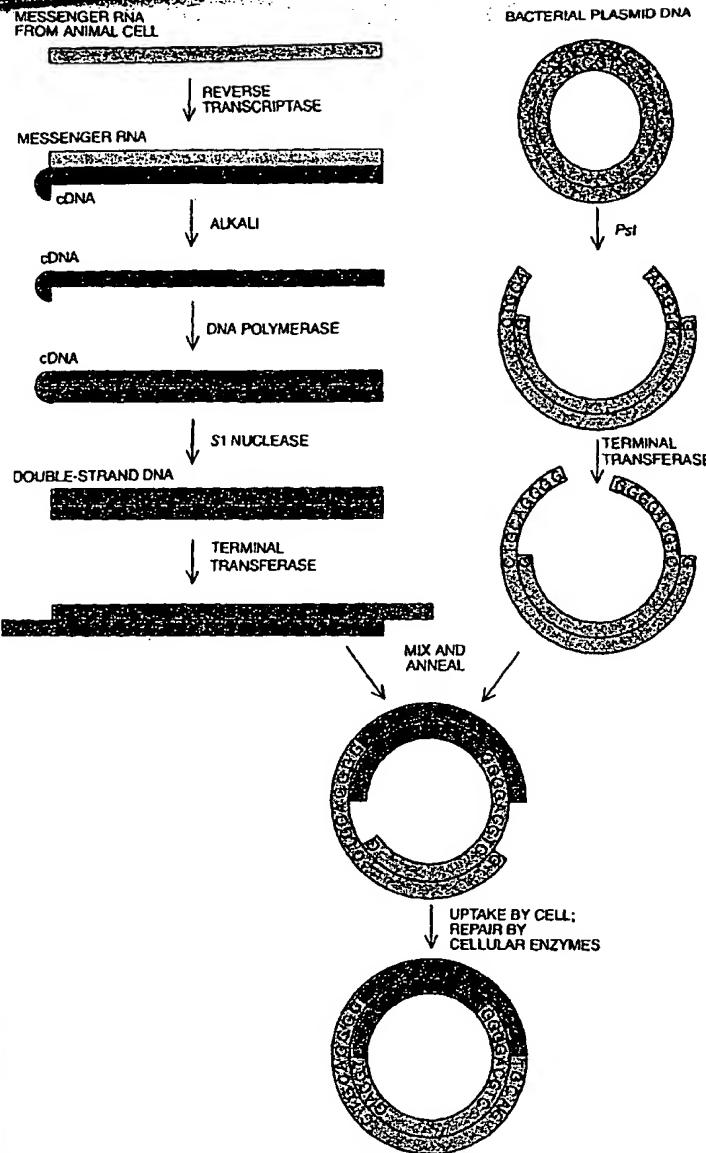
RECOMBINANT-DNA TECHNIQUE for making a protein in bacteria calls for the insertion of a fragment of animal DNA that encodes the protein into a plasmid, a small circular piece of bacterial DNA, which in turn serves as the vehicle for introducing the DNA into the bacterium. The plasmid DNA is cleaved with the appropriate restriction enzyme and the new DNA sequence is inserted into the opening by means of a variety of enzymatic manipulations that connect the new DNA's ends to those of the broken plasmid circle. In the procedure illustrated here, for example, a special enzyme, reverse transcriptase, is first used to copy the genetic information from a single-strand molecule of messenger RNA into a single strand of copy DNA. The RNA template is then destroyed, and a second strand of DNA is made with another enzyme, DNA polymerase. Still another enzyme, S1 nuclease, serves to break the covalent linkage between the two DNA strands. In the next step the double-strand DNA is joined to the plasmid by first using the enzyme terminal transferase to extend the ends of the DNA with a short sequence of identical bases (in this case four cytosines) and then annealing the DNA to the plasmid DNA, to which a complementary sequence of bases (four guanines) has been added. Bacterial enzymes eventually fill the gaps in the regenerated circular DNA molecule and seal the connection between the inserted DNA and the plasmid DNA. The particular plasmid used by the authors to make rat proinsulin in bacteria, designated pBR322, incorporates two genes that confer resistance to two antibiotics: penicillin and tetracycline. The plasmid is cleaved by the restriction enzyme Pst at a recognition site that lies in the midst of the gene encoding penicillinase (the enzyme that breaks down penicillin). The added DNA destroys this enzymatic activity, but the tetracycline resistance remains and is used to identify bacteria containing the plasmid.

long, twisted thread. A bacterium has one millimeter of DNA in a continuous string of some three million bases folded back and forth several thousand times into a space less than a micron (a thousandth of a millimeter) across. In human cells the DNA is packed into 46 chromosomes, each one containing about four centimeters in a single piece, the total amount corresponding to about three billion bases. How can one find and work with a single gene only a few thousand bases long? Fortunately nature has devised certain enzymes (proteins that carry out chemical reactions) that solve part of the problem. These special enzymes, called restriction endonucleases, have the ability to scan the long thread of DNA and to recognize particular short sequences as landmarks at which to cut the molecule apart. Some 40 or 50 of these enzymes are known, each of which recognizes different landmarks; each restriction enzyme therefore breaks up any given DNA reproducibly into a characteristic set of short pieces, from a few hundred to a few thousand bases long, which one can isolate by length.

One can clone such DNA pieces in bacteria. As a first step one purifies the circle of plasmid DNA. The sequences of the plasmids are such that one of the restriction enzymes will recognize a unique site on the plasmid and cut the circle open there. One can insert a chosen DNA fragment into the opening by using a variety of enzymatic techniques that connect its ends to those of the circle. Ordinarily this recombinant-DNA molecule could not pass through the bacterial cell wall. A dilute solution of calcium chloride renders the bacteria permeable, however; in a mixture of treated cells and DNA a few bacteria will take up the hybrid plasmid. These cells can be found among all those that did not take up the DNA if a gene on the plasmid provides a property the bacterium must have to survive, such as antibiotic resistance. Then any bacterium carrying the plasmid will be resistant to the antibiotic, whereas all the others will be killed by it. When one spreads the mixture of bacteria out on an agar plate containing nutrients and the antibiotic, each single bacterium with a plasmid will grow into a separate colony of about 100 million cells. A single colony can be chosen and grown further to yield billions of cells, each of which contains identical copies of the new DNA sequence in a recombinant plasmid.

The Sequencing of DNA

The procedures we have outlined so far are followed in "shotgun" cloning experiments. One breaks up the DNA of an animal cell into millions of pieces and inserts each piece into a different bacterium. In this way a number of collections of all the fragments of human,

mouse, rat and fly DNA have been made. One can determine the structure of any one of these cloned DNA's by breaking up the hybrid plasmid with a restriction enzyme, separating the resulting DNA fragments, determining the base sequence of each of the fragments and then putting the sequences together to deduce the entire structure of the cloned DNA.

There are two methods for sequencing DNA. Both exploit reference points created by restriction-enzyme cleavage of the DNA at a specific short sequence and then work out the rest of the sequence by measuring the distance of each base from that cut. They do this by creating a set of radioactively labeled molecules, each of which extends from the common point to one of the occurrences of a specific base. When these molecules are separated by size and detected by their radioactivity, the length of the smallest one shows the position of the first occurrence of that base; longer molecules correspond to later occurrences. The pattern created by the analysis of these molecules looks like a ladder. From the positions of the rungs one reads off the lengths. By comparing four such patterns one reads off a sequence.

One technique, devised by Allan M. Maxam and one of us (Gilbert), makes use of chemical reagents that detect the different chemical properties of the bases and break the DNA there. To generate the set of fragments the reactions are done for a short time, so that the molecule is broken only occasionally instead of everywhere the base occurs; different molecules will be broken at different places. Four different sets of reagents are used to generate the four patterns. The radioactive label is attached directly to the end of the particular restriction fragment one wants to sequence, so that only the molecules stretching from the labeled end to the break are detected by their radioactivity.

The other sequencing method, devised by Frederick Sanger of the British Medical Research Council Laboratory of Molecular Biology in Cambridge, makes a DNA copy with an enzyme and stops the sequential synthesis, and hence the elongation of the copy, by blocking the movement of the enzyme at a specific base. Here the radioactive label is incorporated into the newly synthesized molecule in four different reactions. Both methods can provide the sequence of from 200 to 300 bases in a single experiment. One of the small plasmids involved in our cloning experiments was sequenced in a year by Gregory Sutcliffe, who worked out the order of the 4,357 bases on one strand and checked them by working out the complementary strand.

Any DNA region carried on a plasmid can be isolated and sequenced. The difficulty is not in determining the sequence but in obtaining the specific

SEQUENCING OF DNA, in the method devised by one of the authors (Gilbert) and Allan M. Maxam, begins with the attachment of a radioactive label to one end of each strand of double-strand DNA (1). The strands of trillions of molecules are separated (2) and a preparation of one of the two kinds of strands is divided among four test tubes (3). Each tube contains a chemical agent that selectively destroys one or two of the four bases A, T, G and C, thereby cleaving the strand at the site of those bases; the reaction is controlled so that only some of the strands are cleaved at each of the sites where a given base appears, generating a set of fragments of different sizes. A strand containing three G's (4), for example, would produce a mixture of three radioactively labeled molecules (5). The reactions break DNA at the G's alone, at the G's and the A's, at the T's and the C's, and at the C's alone. The molecules are separated according to size by electrophoresis on a gel; the shorter the molecule, the farther it migrates down the gel (6). The radioactive label produces an image of each group of molecules on an X-ray film (7). When four films are placed side by side (8), the ladderlike array of bands represents all the successively shorter fragments of the original strand of DNA (9). Knowing what base or pair of bases was destroyed to produce each of the fragments, one can start at the bottom and read off a left-to-right sequence of bases (10), which in turn yields the sequence of the second strand.

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DNA fragments needed. The recombi-
nant-DNA technique serves almost as a
microscope to isolate and to magnify,
by making many copies, a DNA region,
but one does not want to look through a
million bacteria to find a specific gene.
The fundamental problem, which has
no general solution, is to place only
the desired DNA sequence—the desired
structural gene—in a bacterium.

Getting the Right Gene

One straightforward approach is suit-
able for very small proteins. The amino
acid sequence and the genetic code will
predict a sequence of bases that can
specify those amino acids. One can then
chemically synthesize a corresponding
DNA molecule. Exactly this was done
by Keiichi Itakura and his co-workers at
the City of Hope National Medical Cen-
ter in Duarte, Calif., who constructed a
DNA sequence 42 bases long that dic-
tates the structure of somatostatin, a
small hormone consisting of 14 amino
acids. The longer the stretch of DNA,
however, the harder it is to make; the
synthesis of a stretch of DNA 100 bases
long is extremely difficult. Many small
hormones consist of from 50 to 100 ami-
no acids, and enzymes and other pro-
teins range from 200 to several thou-
sand amino acids in length. Further-
more, one does not know the amino
acid sequence of many interesting pro-
teins. (Indeed, the amino acid sequence
of some of these proteins has become
available only through the sequencing
of cloned DNA.)

The desired structural gene is present,
of course, somewhere on the DNA of
the animal cell. The problem is to find
it, but even if that were possible, the
structural information would be broken
up (as we mentioned above) by long
stretches of other DNA. The informa-
tion does exist in a continuous form,
however, on the messenger RNA. More-
over, different cells specialize in the syn-
thesis of different proteins, so that the
appropriate tissue will contain the de-
sired messenger RNA along with other
messengers for the common proteins
made by all cells. Insulin, for example,
is made by the beta cells of the pan-
creas; those cells contain insulin messen-
ger RNA and other cells do not, even
though the insulin gene is present in the
DNA of every cell.

The task is then to convert the desired
structural information from the cell's
messenger RNA into DNA, which can
be cloned. For this one takes advantage
of a special enzyme, reverse transcript-
ase, that can copy a single strand of
RNA to make a complementary strand
of DNA. (The enzyme is found in cer-
tain RNA viruses that reverse the nor-
mal DNA-to-RNA transcription. Such
viruses depend on RNA rather than
DNA to carry their information from
one cell to another and convert the RNA

85

back into DNA with the help of reverse transcriptase after they infect a new cell.) One takes this strand of complementary DNA, called copy DNA, and makes a second strand of DNA with the more usual DNA-copying enzyme. The resulting double-strand cDNA fragments are more or less complete copies not only of the desired messenger RNA but also of all the other messenger RNA's that were present in the tissue. At best, however, only a few of the DNA fragments contain all the wanted structural information. Even in those fragments the regulatory signals that surround the structural sequences refer to translation in the animal cell, not in bacteria, and (since the DNA was made from RNA) there will be no transcriptional commands. Although the cDNA can be cloned, two problems remain: to detect any clones containing the sought-after structural DNA fragment and to provide the appropriate signals.

Finding the Right Clone

It is simple to find the right clone if the experiment began with a pure messenger RNA. One can detect matching sequences by the process called hybridization. The two strands of a DNA molecule can be separated by heating, which breaks the weak bonds that hold the two strands together without breaking the strong chemical bonds between bases along the chain. When a mixture of such strands is cooled, those sequences that match will find each other. The first step of this process is called denatura-

tion, the second step reannealing. The same process serves to identify sequence matches between RNA and DNA.

One grows bacterial colonies on a disk of cellulose nitrate paper, breaks open the bacterial cells where they lie and fixes the released DNA to the paper. When the DNA is denatured and reannealed to radioactive RNA, only the remains of those colonies that contained a plasmid whose sequence matches the messenger become radioactive. Since one keeps a replica (a living duplicate set of the colonies), one can obtain bacteria containing the desired DNA. One grows these bacteria to provide material to identify, in further hybridization tests, other clones that contain the same sequence in different surroundings and may turn out to be more effective in producing the wanted protein.

If one cannot purify the messenger RNA because the specific messenger is a small fraction of all the messengers in a cell, there are other ways to search for the DNA sequence. One useful property is the detailed shape of the corresponding protein molecule. Those shapes that are most different and distinctive can be recognized by the protein molecules called antibodies. Animals make antibodies as part of their protective response to foreign substances. If one injects human insulin into a guinea pig, for example, the guinea pig will make antibodies that bind to human insulin. These antibodies will not bind to guinea pig insulin because they "see" only the shapes that make the human protein different. A purified antibody, then, can

serve as a reagent to detect a particular protein. (This is the way vaccines work. If an animal is injected with an inactivated virus, it is stimulated to make antibodies against the viral proteins. Thereafter the antibodies will protect the animal against infection by that virus by binding to the virus particle and signaling other cells to remove the invader. Without the earlier stimulation the antibody response to the invading virus is too slow to block the infection.)

Even without purifying a specific messenger RNA one can make the RNA molecules function in the test tube by adding the machinery needed to translate the messengers (obtained from the cytoplasm of broken cells) along with radioactive amino acids. Among the small amounts of radioactive proteins that are synthesized one can recognize the protein of interest with antibodies. This provides a means of detecting the presence of a specific messenger. If one takes a recombinant plasmid and hybridizes it to the mixture of RNA's, only the RNA that matches a sequence in the plasmid will anneal to it and therefore no longer function in translation; the plasmid of interest is detected by its ability to block the synthesis of the desired protein. This identification can be verified because the RNA bound to the DNA can be separated from all the other RNA's and then released from the DNA, whereupon it will function to direct the synthesis of the protein.

Regulatory Signals

With these techniques one can clone and identify DNA fragments carrying the information that dictates the structure of a protein. Will the information work in bacteria?

One must provide regulatory signals the bacterium can use. One of them is the signal to start the synthesis of a messenger RNA; in bacteria it is a region of DNA immediately in front of the segment of DNA that will be transcribed into RNA. The second important signal functions as part of the messenger RNA, telling the bacterial translation machine to "Start here." All bacterial genes have these two kinds of start signals (some of which work better than others). They also have two stop signals, one for translation and one for transcription. A simple way to make the new protein sequence is to cut a bacterial gene open in its middle with a restriction enzyme and to insert the new DNA there. This results in a hybrid protein that starts out as some bacterial protein and then continues as the string of amino acids one wants. That is how the chemically synthesized gene for somatostatin was made to work in bacteria. The DNA for those 14 amino acids, followed by a stop signal, was inserted near the end of a 1,000-amino-acid protein. After the bacterium made the hybrid protein the
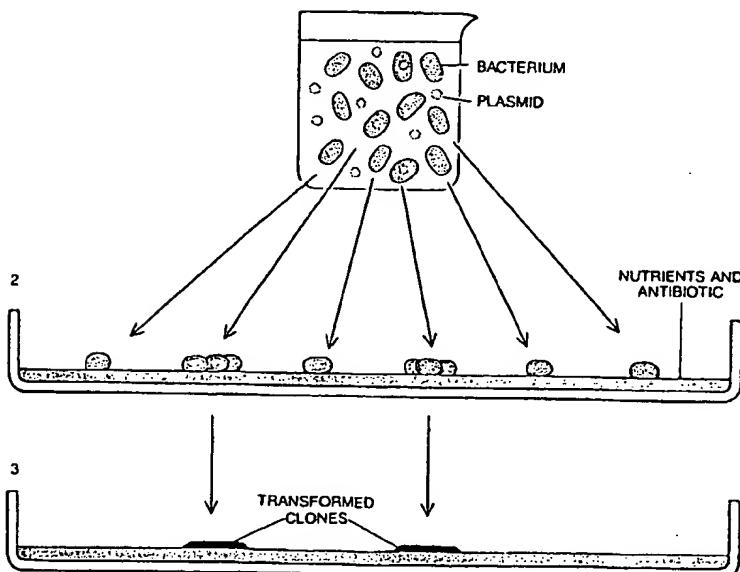
RECOMBINANT PLASMIDS (color) bearing the inserted animal-protein genes and genes for resistance to tetracycline are mixed with bacteria (1). Some cells take up the plasmid. The mixture of cells is spread on a culture medium containing the antibiotic (2), which kills all the cells that do not have the plasmid. The cells that have taken up the plasmid are antibiotic-resistant; they live, and each of them gives rise to a clone, a colony of genetically identical cells (3).
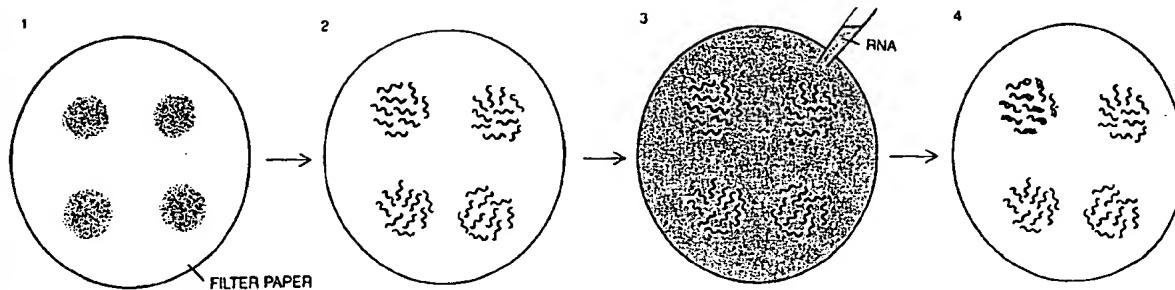
CLONE CONTAINING DESIRED DNA can be found among all the successfully transformed clones (*1*) by means of RNA-DNA hybridization if one has a pure messenger-RNA probe for the desired sequence. The cells are broken open and their DNA is denatured and fixed to filter paper (*2*). The RNA probe (RNA molecules labeled with a radioactive isotope) is added (*3*). The RNA (*color*) will anneal to any DNA whose sequence it matches, forming RNA-DNA hybrids (*4*); the remainder of the RNA is washed away. The presence of the hy-

somatostatin part was cleaved off chemically and purified.

Not only can the bacterial gene serve to provide the regulatory signals but also it may endow the hybrid protein with further useful properties. For example, a few bacterial proteins are secreted through the membrane that surrounds the cell. If one inserts the animal DNA into the gene for such a protein, the bacterial part of the hybrid protein will serve as a carrier to move the new protein through the membrane so that it is more easily observed and purified.

We exploited all the techniques described above to obtain a copy of the insulin gene and to insert it into bacteria to make proinsulin. Insulin is a small hormone made up of two short chains, one chain 20 amino acids long and the other 30 amino acids long. These two chains are initially part of a longer chain

of 109 amino acids, called preproinsulin. As preproinsulin is synthesized in the beta cells of the pancreas, the first 23 amino acids of the chain serve as a signal to direct the passage of the molecule through a cell membrane. As this happens those amino acids are cleaved off, leaving a chain of 86 amino acids: proinsulin. The proinsulin chain folds up to bring the first and last segments of the chain together, and the central portion is cut out by enzymes to leave insulin. The role of the central portion is to align the two chains comprising insulin correctly. If the two chains are taken apart later, they do not reassemble easily or efficiently. (In spite of these difficulties Itakura and his co-workers synthesized two DNA fragments corresponding to the two chains of human insulin and attached them separately, like somatostatin, to the same large bacterial gene in

order to synthesize two separate hybrid proteins in two different bacteria. Then they cut off the two short pieces, purified them and put them together to form insulin.)

The Proinsulin Experiment

In our experiments we started with a tumor of the insulin-producing beta cells of the rat. (We worked with rat insulin because at the time we began our experiments the guidelines established by the National Institutes of Health for recombinant-DNA investigations would not allow us to insert the human insulin gene into bacteria; that prohibition has since been removed.)

We made DNA copies of the beta-cell messenger RNA and put them into a plasmid, in the middle of a gene for a bacterial protein, penicillinase, that
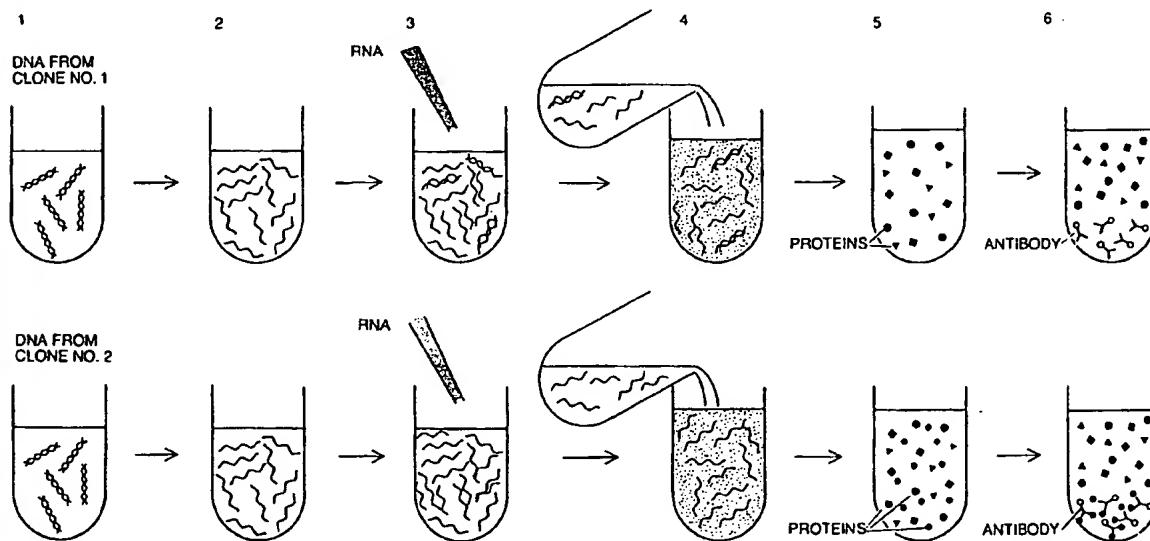
HYBRID-ARRESTED TRANSLATION, a technique developed by Bryan Roberts of the Harvard Medical School, identifies a clone (*top*) containing the desired DNA even in the absence of a purified RNA probe. DNA from clones being tested (*1*) is denatured (*2*). Unpurified RNA (the same RNA used to make the inserted DNA) is added (*3*); it anneals to any matching DNA. Placed in a "translation system" con- taining radioactively labeled amino acids (*4*), the unhybridized RNA directs the synthesis of radioactive proteins, but the hybridized RNA cannot be translated; the specific protein (*color*) encoded by the desired DNA is not synthesized in the presence of the clone containing that DNA (*5*). The presence or absence of that protein is determined by an antibody test. Antibody to the protein, fixed to plastic beads,
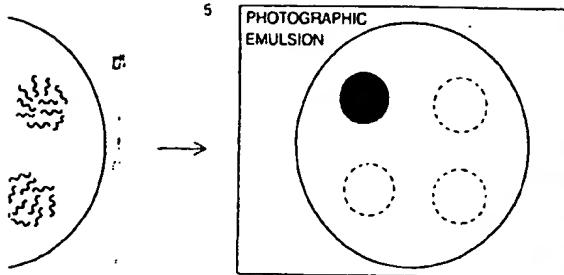
brids is revealed by autoradiography: a photographic emulsion is placed on the filter paper and after exposure the clone containing the desired DNA is identified as a dark spot (5).

would be secreted through the membrane of the bacterial cell. We looked among the bacterial colonies by hybridization, we proved that we had the right hybrid plasmid by blocking the synthesis of insulin in a test tube as we described above and we sequenced the DNA to see exactly what part of the insulin gene we had. Once we had found one hybrid plasmid, we used it to find 48 more by repeating the hybridization test. These 48 clones represented 2 percent of all the clones we had made.

Would any of those clones actually synthesize insulin? We looked among the clones containing insulin DNA for any that were synthesizing a hybrid protein part of which was proinsulin. For this we relied on a sensitive radioactive-antibody test. We coated plastic disks with antibody directed against either insulin or penicillinase and exposed them
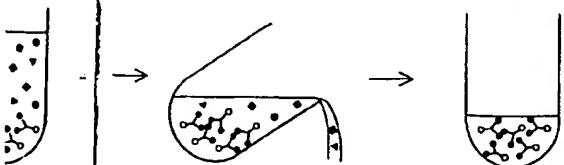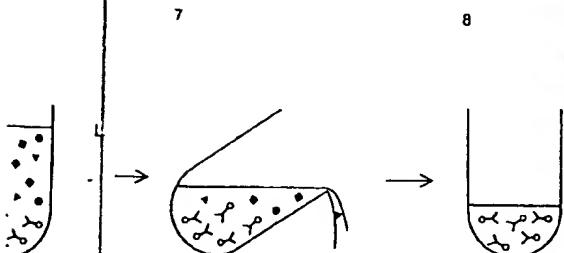
is added and binds the protein, precipitating the protein out of the solution (6), which is poured off (7). Measurement of the precipitates' radioactivity (8) shows that one clone (top) contains the desired DNA, because it blocked the synthesis of the specific protein.

to the contents of cells from each clone. Any insulin (or penicillinase) present in the cells binds to the antibody and is thereby fixed to the plastic disks. Then we applied radioactively labeled anti-insulin antibody to detect the presence of proteins with insulin shapes. One clone gave positive responses, both on disks coated with anti-insulin and on those coated with antipenicillinase, to radioactive antibody to insulin, thereby demonstrating the presence of a penicillinase-insulin hybrid protein.

To see if the bacteria were secreting the hybrid protein we grew the clone in liquid culture and tried to extract the protein by a method that does not burst the bacterial cell membrane. The test showed the fused protein to be present outside the membrane: it was secreted, as we had hoped it would be.

Sequencing the DNA showed that the DNA fragment and the details of the fusion were such that the structural information in the clone was only for proinsulin and did not contain the "pre" region. In order to make insulin we removed most of the bacterial protein and the middle segment of the proinsulin with the digestive enzyme trypsin. Would the insulin made from the bacteria be an active hormone? Stephen P. Naber and William L. Chick of the Elliot P. Joslin Research Laboratory in Boston tested the molecule by showing that it affected the metabolism of sugar by fat cells, as it should.

Improving the Yield

The amount of proinsulin made by the original clone was very small; we are currently engaged in various manipulations to improve the yield. Regulatory signals must be not only efficient but also optimally placed. One need not be satisfied with the signals that happen to surround preexisting bacterial genes. With restriction enzymes one can clip out small DNA fragments that carry only the regulatory signals and tie them together with a DNA-linking enzyme to make new combinations. One can trim back the ends of these fragments by nibbling off bases with still other enzymes before reconnecting them. This will alter the spacings between the signals and the structural sequence. Although each of these manipulations generates only a small number of correct molecules, by cloning after each step one can make large amounts of the DNA and work out its sequence, and then continue the tinkering.

Moreover, one can synthesize short desired DNA sequences and tie them to other fragments. For example, David V. Goeddel and his co-workers at Genentech, Inc., took a piece of DNA containing the structural information for human growth hormone (168 amino acids), connected it to a synthetic piece of DNA containing part of the translation-

al start signal and attached that combination in turn to a fragment containing the rest of the regulatory signals. When this DNA construction was cloned, the bacteria made a protein of the shape (as recognized by antibodies) and size of growth hormone (although not yet with demonstrated hormone activity).

Although we do not yet know the optimal combinations of the DNA elements for making insulin in bacteria, finding them is only a matter of time. There are other problems to be considered. Often the new animal proteins are broken down in the bacterial cell because their structure is such that enzymes normally present in the bacteria can digest them. Ways have to be found to stabilize the proteins either by removing these enzymes, by embedding the new protein in a hybrid protein to protect it or by secreting it from the cell. Messenger-RNA molecules themselves are often unstable within the cell; modifications in their structure and in the cell itself can make them more effective and lead to increased protein synthesis. And if the number of copies of the plasmid carrying the gene in each cell can

be increased, more of the product will be made.

While we work to improve the yield of rat proinsulin and to purify it we expect to apply the same methods to the bacterial synthesis of human insulin. Investigators in other laboratories are also working on the problem, and one can hope that eventually the manufacture of human insulin by bacteria will be cheaper than the purification of insulin from pigs and cattle, the present sources of the hormone. Clearly other human hormones can also be prepared by these procedures. What other therapeutic proteins might be made in bacteria? In general any human protein that cannot be obtained in useful form from animals is an excellent prospect.

Other Proteins from Bacteria

Many genetic diseases are caused by the lack of a single protein. Replacement therapy may be possible if such proteins can be made in bacteria. Vaccines against viral or parasitic infections are a further wide class of possibilities. Today in order to make a vaccine one
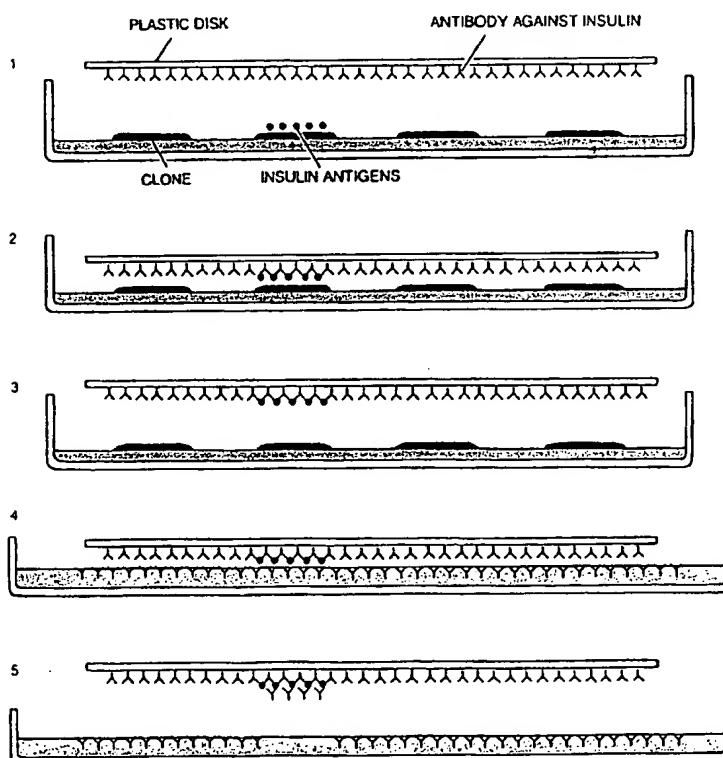
RADIOACTIVE-ANTIBODY TEST, developed by Stephanie Broome and one of the authors (Gilbert), is used to search among the bacterial clones containing insulin DNA for signs that insulin is indeed being synthesized. A plastic disk coated with an anti-insulin antibody is first exposed to the contents of cells from each clone (*1*). Any insulin present in the cells is bound to the antibody (*2*) and thereby fixed to the plastic disk (*3*). Radioactively labeled antibody (color) to insulin is then applied to the disk in order to detect the presence of the protein (*4*, *5*). When the test is repeated with a plastic disk coated with an antipenicillinase antibody, only a hybrid protein, part penicillinase and part insulin, will bind the labeled antibody.

93

must be able to grow the disease organism in large amounts; often this is impossible or dangerous. Furthermore, the vaccine must be rendered harmless before it is administered, which can be difficult. The new technology offers the chance to make in bacteria only the protein against which the antibody response needs to be directed. This would eliminate any need to work with the intact disease organism. For example, the hepatitis *B* virus, which causes serum hepatitis, cannot be grown outside the body. The only source of this small DNA virus is the blood of infected human beings. The DNA of the virus has now been cloned in several laboratories and its complete sequence has been worked out, revealing the structure of the viral proteins; now the proteins are being made in bacteria. A flood of new information has resulted from this work.

A particularly promising candidate is interferon, a protein cells make to block viral infections quickly. (The antibody response is much slower.) Interferon appears to be the body's first line of defense against viruses. It may also have a therapeutic effect in some cancers. Interferon has never been available in sufficiently large amounts, however, to determine how effective it might really be in protecting against disease. The ability to test the activities of human interferon will soon be a reality because the protein has now been made in bacteria. Weissmann, with his colleagues Shigekazu Nagata, Hideharu Taira, Alan Hall, Lorraine Johnsrud, Michel Streuli, Josef Ecsödi and Werner Boll, along with Kari Cantell of the Finnish Red Cross, applied many of the techniques we have described to clone and to express this protein. The problem they faced was that the messenger RNA for interferon is far rarer than the one for insulin, even in white blood cells that have been stimulated by infection with a virus to make interferon. They took messenger RNA from these white blood cells (17 liters at a time), made double-strand cDNA and cloned it by the procedures we have described.

They looked through some 20,000 clones (in batches) by hybridizing the plasmid DNA from the clones to the messenger RNA of the white blood cells, isolating the RNA that annealed and checking the RNA to see if it was able to direct the synthesis of interferon (not in the test tube but by injecting the RNA into a particularly large cell, a frog's egg). Fortunately interferon is a remarkably potent substance, and so the amount synthesized in the frog's egg could be detected by its ability to protect cells against viruses.

Once Weissmann and his colleagues had found a batch of clones that could hybridize to interferon messenger RNA they tested progressively smaller groups of those clones to find the correct one. Then, with that clone as a probe, they found other clones by means of hybridization testing. Finally they tested extracts of the bacteria carrying the interferon DNA (inserted into the penicillinase gene) directly to see if any of the bacterial clones made biologically active interferon. A number of clones did, confirming that the interferon structural DNA had been correctly identified. The sequencing of the DNA of those clones will determine the structure of interferon, which is still not known.

The amount of interferon made in the bacteria was extremely small: only one or two molecules per cell. (Bacterial proteins are usually made in from 1,000 to 100,000 copies per cell.) We are confident that the methods we have described will solve this problem and lead to the production of enough interferon for clinical tests.

The Recombinant-DNA Debate

The development of the genetic-engineering techniques described in this article was greeted, over the past decade, with both excitement and alarm. The possible benefits of the techniques were obvious, but some people felt there was reason for concern. Biologists called for an evaluation of the possible hazards of this research; the result was an unprecedented national and international effort in which the public, governments and the scientific community joined to monitor research activities. New knowledge about the properties of genes and the behavior of the bacteria used in this work (usually *Escherichia coli*) has led to a steady lessening of these concerns and to a relaxation of the guidelines that once restricted such experiments. In retrospect, with the advantage of hindsight, the concerns about hypothetical hazards seem to have been unwarranted. We know of no adverse effects from this research. The great potential of the new techniques, both in promoting the growth of basic knowledge and in making possible the synthesis of products of direct benefit to society, is much closer to realization than seemed likely only a few years ago.
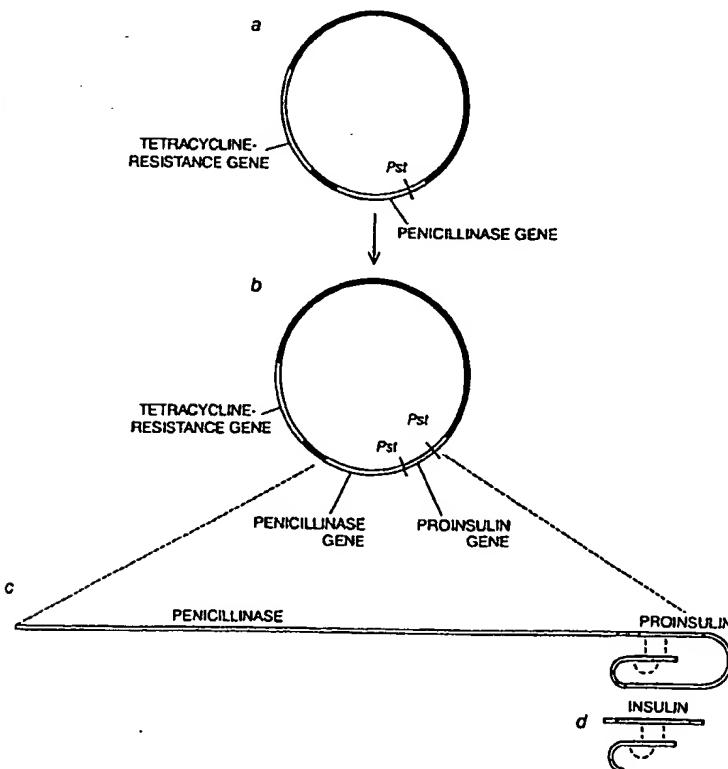
RAT INSULIN WAS OBTAINED by the authors from a hybrid protein composed of part of the bacterial penicillinase molecule and a molecule of proinsulin, an insulin precursor. The map of the plasmid that served as a vehicle, *pBR322* (*a*), shows the location of the genes for the two enzymes conferring antibiotic resistance and the site of cleavage by the restriction enzyme *Pst*. The next map (*b*) shows the structure, as determined by DNA sequencing, of the recombinant plasmid in the bacterial clone that synthesized proinsulin. The proinsulin sequence (*color*) lies between two *Pst* sites that were regenerated in the insertion process. The hybrid protein synthesized by the clone (*c*) comprises most of the penicillinase and also the proinsulin molecule (*color*); broken lines represent disulfide bonds. The authors cut away most of the penicillinase and the middle segment of the proinsulin (*light color*) to make biologically active insulin (*d*).

94

Regulation of Melanogenesis by Human Uveal Melanocytes in vitro

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The purpose of this study was to investigate factors regulating melanogenesis in cultured human uveal melanocytes. The effects of various substances on the melanin content, tyrosinase activity and growth of cultured uveal melanocytes were tested. 12-O-tetradecanoyl-phorbol-13-acetate (a protein kinase C activator) and various cAMP-elevating agents, including isobutylmethylxanthine, cholera toxin, and dibutyryl-cAMP increased melanin content per culture, tyrosinase activity and cell numbers of uveal melanocytes in a dose dependent manner. Basic fibroblast growth factor (tyrosine kinase activator) stimulated growth but did not affect melanin content per culture of uveal melanocytes *in vitro*. These results indicate that cAMP-elevating agents and protein kinase C activator stimulate melanogenesis and growth of cultured uveal melanocytes. Tyrosine kinase activator stimulates growth but not melanogenesis of cultured uveal melanocytes. © 1997 Academic Press Limited

Key words: uveal melanocytes; melanogenesis; basic fibroblast growth factor; phorbol ester; cAMP-elevating agents.

1. Introduction

Melanin has a variety of important functions including a barrier function against visible light and ultraviolet irradiation (UVR), a potential scavenger of cytotoxic radicals and their intermediates, and possible participation in the development of neural tissues (Hearing and King. 1993). Melanin contained in uveal melanocytes (UM) may play an important protective role in the prevention of certain eye diseases related to UVR and visible light, (Gallagher et al., 1985; Tucker et al., 1985; Weiter et al., 1985; 1986; Sarna, 1992; Tayler et al., 1988, 1992; Cruickshanks, Klein and Klein, 1993) and may be related to the pathogenesis of some autoimmune eye diseases (Sakamoto, Murata and Inomata, 1991; Broekhuyse et al., 1993; Bara et al., 1995). The binding of various drugs by ocular melanin can affect their therapeutic and toxicological activities (Barber and Smith, 1957; Salazar-Bookaman. Wainer and Patel, 1994).

Despite the importance of uveal melanin, little is known about melanogenesis by UM in vivo or in vitro (Mund. Rodrigues and Fine, 1972; Endo and Hu, 1973; Laties, 1974; Dryja et al., 1978; Waldrep and Kaplan, 1986; Hu, Teramura and Mah, 1987; Eagle, 1988; Schraermeyer, 1993; Goodall et al., 1994).

We have developed methods for isolation and cultivation of human UM and have established many pure cell strains (Hu et al., 1993a). Our previous work has documented that cultured human UM produce melanin in vitro, and that cultured UM from eyes of different iris color expressed different capacities for melanogenesis in vitro. Therefore, cultured UM are an excellent model system for studying melanogenesis by UM (Hu et al., 1995). The growth of cultured UM could be regulated by growth factors (such as basic fibroblast growth factor, bFGF), cAMP-elevating agents and protein kinase C (PKC) activators (Hu et al., 1993b). In this study, the effects of various factors on melanogenesis by UM in vitro were investigated, and the significance of these results are discussed.

2. Materials and Methods

Reagents

F12 nutrient mixture (F12 medium), fetal bovine serum (FBS), geneticin, L-glutamine, gentamicin, trypsin solution, and trypsin-ethylene diaminetetraacetic acid (trypsin-EDTA) solution were obtained from Gibco (New York, NY, U.S.A.). 12-O-tetradecanoyl-phorbol-13-acetate (TPA), synthetic melanin (prepared by oxidation of tyrosine with hydrogen peroxide), dibutyryl-cAMP (dbcAMP), cholera toxin (CT), and isobutylmethylxanthine (IBMX) were obtained from Sigma (St. Louis, MO, U.S.A.).

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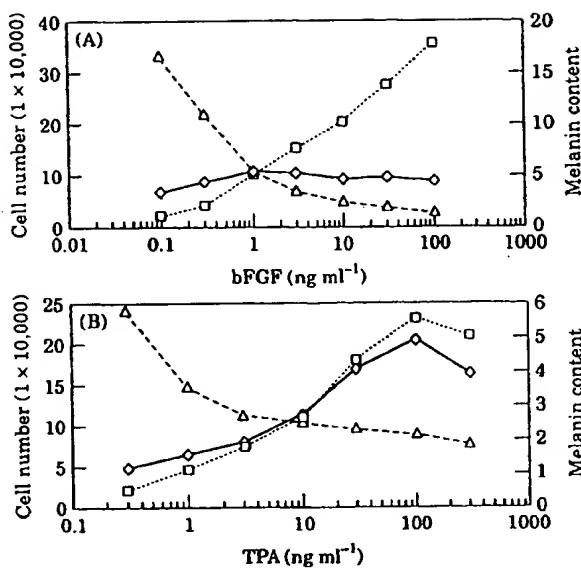
FIG. 1. Effects of bFGF and TPA on melanogenesis and growth of uveal melanocytes (UM). Cultured human UM were plated into the 24 well plates. Various concentrations of bFGF (A) and TPA (B) were added to the bFGF/TPA deleted medium for testing. After 6 days of culture, the cells were detached and counted. Melanin content was measured and expressed as melanin content per well or melanin content per cell. Data are averages from three wells of each group. □, Cell number; △, Melanin/cell (10 pg); ◇, Melanin/well (μg).

Human recombinant bFGF was obtained from Promega (Madison, WI, U.S.A.).

Cell Culture

Human UM were isolated from adult autopsy eyes as previously reported (Hu et al., 1993a). The isolated UM were cultured in Falcon dishes (Becton Dickinson, Oxnard, CA, U.S.A.) with FIC medium, which is F12 medium supplemented with 10% FBS, 2 mM glutamine, 20 ng ml⁻¹ bFGF, 0·1 mM IBMX, 10 ng ml⁻¹ CT, and 50 μg ml⁻¹ gentamicin. Geneticin, a cytotoxic agent, was added (100 μg ml⁻¹) for 3–7 days when necessary to eliminate contaminating cells. After reaching confluence, the UM were detached using trypsin-EDTA solution, diluted 1:3–1:6, and subcultured.

Experiments

Melanin content and growth capacity The UM used in this study consisted of two cell lines isolated from the choroid and one cell line isolated from the iris. These cells had been in culture for no longer than 2 months and had been passaged three to six times at a dilution of 1:3–1:4. The purity of the cell lines was demonstrated by immunocytochemical methods. UM display S-100 antigen but not cytokeratin, whereas pigment epithelial cells display both proteins, and fibroblasts display neither of these proteins (Hu et al., 1993a).

The UM were plated into 24-well plates (Corning Glassworks, Corning, NY, U.S.A.) with FIC medium at

a density of 2 x 10⁴ cells per well. After 24 hr, the FIC medium was replaced with 0·5 ml of the test medium. The media were replaced every 3 days, and the cells were cultured for 6 days.

Two base media were used to test the effect of various agents:

(1) bFGF/TPA deleted medium. The base medium consisted of F12 medium with 10% FBS, 10 ng ml⁻¹ CT, 0·1 mM IBMX, and 2 mM glutamine. bFGF (0·1, 0·3, 1, 3, 10, 30, 100 ng ml⁻¹) was added to test the effects. Each of the supplemented media were added to UM cultures in triplicate. bFGF/TPA deleted medium without supplements was used in three wells as a control. To test the effect of TPA, the culture medium was changed to TIC medium (which consisted FIC medium with 50 ng ml⁻¹ TPA substituted for bFGF) and cells were cultured for 3 days. Cells were plated into 24-well plates with TIC medium. After 24 hr, the TIC medium was replaced with bFGF/TPA deleted medium. TPA (0·3, 1, 3, 10, 30, 100, 300 ng ml⁻¹) were added to test its effect.

(2) cAMP deleted medium. The base medium consisted of F12 medium with 10% FBS, 20 ng ml⁻¹ bFGF, and 2 mM glutamine. Various cAMP-elevating agents were added to test their effects. These included dbcAMP (0·03, 0·1, 0·3, 1, 3 mM), IBMX (0·001, 0·01, 0·1, 1 mM), and CT (1, 10, 100, 1000 ng ml⁻¹). Each of the supplemented media was added to UM cultures in triplicate. The cAMP deleted medium without supplemental cAMP-elevating agents was used in three wells as a control.

After 6 days, the cells were detached with trypsin-EDTA solution for cell counting and melanin measure-
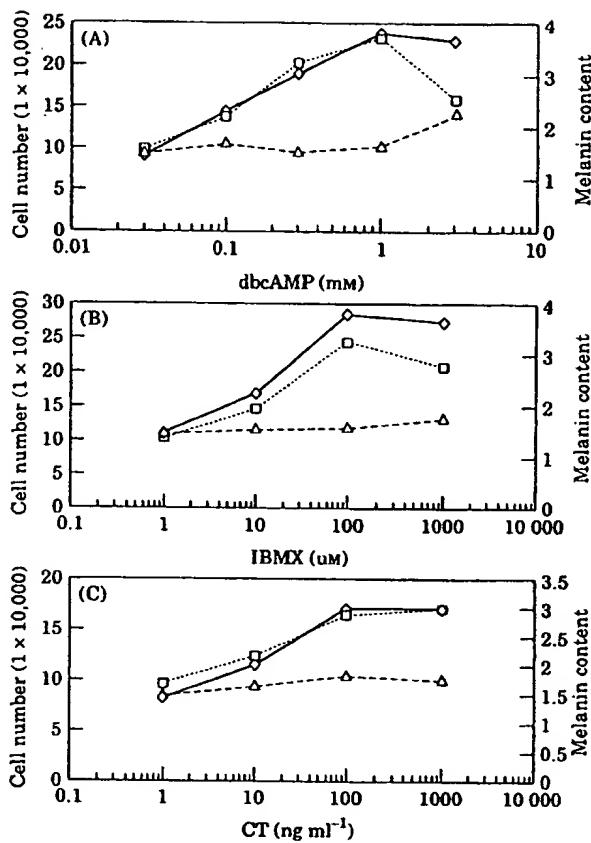
FIG. 2. Effect of IBMX, CT and dbcAMP on melanogenesis and growth of UM. Cultured UM were plated into the 24-well plates. Various concentrations of dbcAMP (A), IBMX (B) and CT (C) were added to the cAMP deleted medium for testing. After 6 days of culture, the cells were detached and counted. Melanin content was measured and expressed as melanin content per well or melanin content per cell. Data are averages from three wells of each group. □, Cell number; △, Melanin/cell (10 pg); ◇, Melanin/well (μg).

ment. Cells were counted in a hemocytometer. Cell suspensions were centrifuged, and the pellet was dissolved in 1 N NaOH. Melanin concentration was determined by measurement of optical density at 475 nm and compared with a standard curve determined using synthetic melanin. Melanin content was expressed as ng cell^{-1} and μg culture^{-1} (Hu et al., 1995).

Tyrosinase activity. The study of the effects of various factors on tyrosinase activities was the same as that for the study of melanin content with some exceptions: (1) UM were plated into 35 mm culture dishes at a density of 6 × 10^4 cells per well. (2) The concentrations of various test substances were: bFGF (0, 1, 20 ng ml^{-1}), TPA (0, 3, 30 ng ml^{-1}), dbcAMP (0, 0·1, 1·0 mM), IBMX (0, 0·1, 1·0 mM) and CT (0, 1·0, 100 ng ml^{-1}). (3) Tyrosinase activity was measured after 6 days in culture.

Tyrosinase activity was evaluated using an adaptation of the Pomerantz method (Pomerantz, 1966; Orlow et al., 1990), which is based on the measurement of ^3H_2O released by the enzymatic hydroxylation of tyrosine, and is expressed in units (a unit of

tyrosinase activity was defined as the activity of enzyme that catalysed and hydroxylation of 1 pM of tyrosine/mg protein per hour).

Student's *t* test was used to assess statistical significance.

3. Results

(1) bFGF/TPA-like action: UM did not survive when cultured with bFGF/TPA deleted medium alone. The cells degenerated and detached from the wells after several days in culture. UM grew well when bFGF or TPA was added.

bFGF stimulated the growth of UM and decreased melanin content per cell in a dose dependent manner [Fig. 1(A)]. The number of cells per well increased significantly and melanin content per cell decreased significantly in bFGF concentrations ranges from 0·1–100 ng ml^{-1}. The difference of melanin content per cell between each level of bFGF was statistically significant ($P < 0·01$). No significant difference in the content of melanin per culture was demonstrated in the ranges of concentrations of bFGF tested ($P > 0·05$) [Fig. 1(A)]. There was no statistically significant

differences in cell number, melanin content per culture or melanin content per cell between the lowest concentration of bFGF tested (0·1 ng ml⁻¹) and culture medium without bFGF ($P > 0.05$).

TPA stimulated the growth and increased melanin content per culture in a dose-dependent manner with concentrations of TPA ranging from 0·3–100 ng ml⁻¹, a slight decrease of the growth and melanin content per cell occurred in with a concentration of TPA of 300 ng ml⁻¹ [Fig. 1(B)]. The difference in melanin content per culture between each level of TPA was statistically significant ($P < 0.01$ between 3 and 10 ng ml⁻¹, 10 and 30 ng ml⁻¹, 30 and 100 ng ml⁻¹ and 100 and 300 ng ml⁻¹ and $0.01 < P < 0.05$ between 0·3 and 1 ng ml⁻¹ and 1 and 3 ng ml⁻¹). A decrease in melanin content per cell was seen in the lowest ranges of concentration, but the difference between the various groups were not statistically significant ($P > 0.05$). Differences of cell number and melanin content per culture or per cell between the lowest concentration of TPA tested (0·3 ng ml⁻¹) and culture medium without TPA were not statistically significant ($P > 0.05$).

(2) cAMP action: UM cultured with medium without cAMP-elevating agents grew slowly. Addition of CT, IBMX or dbcAMP resulted in significant stimulation of growth and an increase in melanin content per culture (Fig. 2). These effects were dose-dependent within certain ranges (dbcAMP) from 0·03–1·0 mM. IBMX from 0·001–0·1 M and CT from 1–100 ng ml⁻¹). The difference in melanin content per culture between various concentrations of these cAMP-elevating agents were statistically significant. Concentrations higher than the optimal levels (dbcAMP: 1·0 mM; IBMX: 0·1 mM; and CT: 100 ng ml⁻¹) usually inhibited growth significantly, but melanin content remained unchanged. No significant difference of content in melanin per cell could be found at the various concentrations tested. Differences in cell number, melanin content per culture or per cell between the lowest concentration of dbcAMP (0·03 mM), IBMX (0·001 mM), or CT (1·0 ng ml⁻¹) and culture medium without cAMP-elevating agents were not statistically significant ($P > 0.05$).

(3) Tyrosinase activity: Tyrosinase activity of UM cultured with 1 ng ml⁻¹ and 20 ng ml⁻¹ of bFGF were slightly less than UM cultured with bFGF/TPA deleted medium ($P > 0.05$ at 1 ng ml⁻¹, $0.01 < P < 0.05$ at 20 ng ml⁻¹) [Fig. 3(A)].

Tyrosinase activities of UM cultured with 3 ng ml⁻¹ and 30 ng ml⁻¹ of TPA were significantly higher than UM cultured with bFGF/TPA deleted medium ($P < 0.01$ at 30 ng ml⁻¹, $0.01 < P < 0.05$ at 3 ng ml⁻¹ [Fig. 3(A)].

Tyrosinase activities of UM cultured with IBMX and dbcAMP were significantly higher than those cultured without cAMP-elevating agents ($P < 0.01$ at IBMX 0·1 mM and 1·0 mM, and at dbcAMp 1·0 mM; $0.01 < P < 0.05$ at dbcAMP 0·1 mM) [Fig. 3(B)]. Tyrosinase
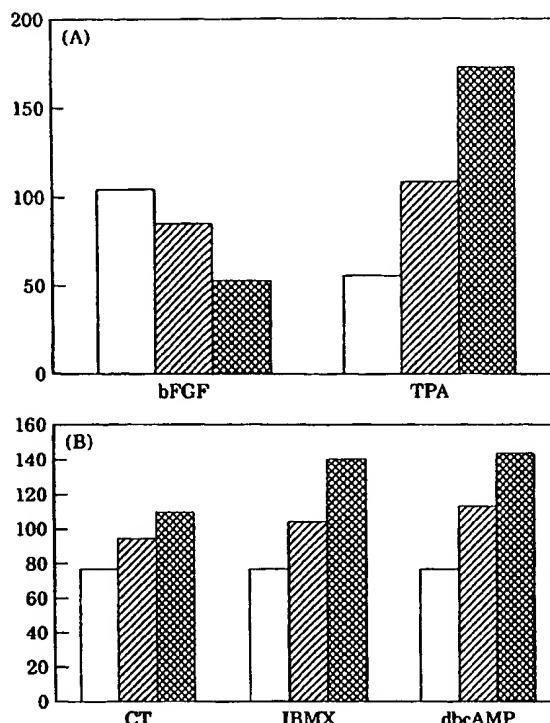
FIG. 3. Effect of bFGF and TPA (A) and various cAMP elevating agents (B) on tyrosinase activity of cultured UM. Cultured human UM were plated into 35 mm dishes. Various concentrations of bFGF (0, 1·0, 20 ng ml⁻¹) and TPA (0, 3·0, 30 ng ml⁻¹) were added to the bFGF/TPA deleted medium and various concentrations of cAMP elevating agents were added to the cAMP deleted medium (dbcAMP and IBMX: 0, 0·1, 1·0 mM; CT: 0, 1·0, 100 ng ml⁻¹). After 6 days of culture, the cells were detached. Tyrosinase activity was measured by an adaptation of Pomerantz method, and expressed at units (a unit of tyrosinase activity was defined as the activity of enzyme that catalysed and hydroxylation of 1 pM of tyrosine/mg protein per hour). ☐, 0; ▨, Low concentration; ▦, High concentration.

activities of UM cultured with CT (1·0 ng ml⁻¹ and 100 ng ml⁻¹) were also higher than those cultured without cAMP-elevating agents, but the differences were not statistically significant ($P > 0.05$) [Fig. 3(B)].

4. Discussion

Abnormalities of quantity or quality of uveal melanin occur in a number of pathologic processes (Hu, 1983; Green, 1986; King, Townsend and Otteing, 1993; Orlow, 1994). Development of certain eye diseases may be related to exposure to visible and UV light, such as age-related macular degeneration, senile cataract and intraocular melanoma. Uveal pigmentation could protect intraocular tissues from irradiation and may play a role in the prevention of such disorders (Gallagher et al., 1985; Tucker et al., 1985; Weiter et al., 1985, 1986; Sarna, 1992; Tayler et al., 1988, 1992; Cruickshanks et al., 1993). Purified uveal melanin can induce experimental autoimmune uveitis and may play a role in the

pathogenesis of acute anterior uveitis. sympathetic ophthalmia and Vogt-Koyanagi-Harada syndrome (Sakamoto et al., 1991; Broekhuyse et al., 1993; Bara et al., 1995). Accumulation of various drugs by ocular melanin could govern their therapeutic and toxicological activities (Barber and Smith, 1957; Salazar-Bookaman et al., 1994).

Although melanin of UM may have an important role in the physiology, pharmacology, pathology and toxicology of the eye, little is known about factors regulating melanogenesis by UM in vivo or in vitro. Controversy exists concerning the melanogenic and growth capacities of adult UM in vivo (Mund et al., 1972; Endo and Hu, 1973; Laties, 1974; Dryja et al., 1978; Eagle, 1988; Schraermeyer, 1993; Goodall et al., 1994).

In experimental animals, tyrosinase activity and melanosomes in early stages of maturation (Stage II and III) have been found in the iris of some adolescent animals, indicating that some degree of melanogenesis and turnover of melanin occurs in the eye of some adult animals (Endo and Hu, 1973; Laties, 1974; Schraermeyer, 1993; Varela et al., 1995).

In humans, iridal pigmentation is generally assumed to be stable past infancy. However, careful review of follow up data of eye color on a group of twin pairs accumulated in the Louisville Twin Study reveals that, in 10–20% of individuals, the extent of iridial pigmentation remains variable through adolescence (Carino et al., 1994). Therefore, the change of uveal pigmentation may be more frequent than once supposed.

Since the first method for culturing human epidermal melanocytes (EM) was conceived, many investigators have used these cells as a model to study factors regulating melanogenesis of EM (DeLuca et al., 1988; Herlyn et al., 1988; Bennett, 1989; Gordon, Mansur and Gilchrest, 1989; Brooks, Birch and Hart, 1990; Halaban and Moellmann, 1990; Halaban et al., 1993).

UM differ from EM in many respects. EM continuously synthesize and transfer melanin to keratinocytes, and keratinocytes elaborate mitogens which stimulate the growth of EM. There is no definite evidence indicating that normal adult UM actively transfer melanin in vivo, and the UM remain relatively stationary throughout life. In ocular albinism, depigmentation occurs in the eye but not in the skin (King et al., 1993), indicating that melanogenesis of UM involves a pathway different from that of the skin. Therefore, independent investigations of UM are necessary.

We have developed a method for isolation and cultivation of pure cultures of human UM (Hu et al., 1993a). UM could be maintained in special culture medium for long periods with 40–50 divisions. Cultured UM produced melanin and expressed tyrosinase activity in vitro. UM from eyes of different iris colors expressed different capacities for melanogenesis

in vitro (Hu et al., 1995). Therefore, cultured UM could be an excellent model for studying melanogenesis by UM.

In the present studies, three parameters (melanin content per cell, melanin content per culture and tyrosinase activity) were used to evaluate the effect of various substances on melanogenesis in cultured UM. An important concept for consideration is which parameter is most meaningful to evaluate melanogenesis of UM in vitro. Tyrosinase activity is an important factor, but not the sole factor in determining the rate of melanin production (Hu et al., 1995). Measurement of melanin content is more direct and meaningful. Melanin content per culture is more important than melanin content per cell for two reasons. First, melanin content per cell in cultured UM is not only determined by the production of melanin, but also by the growth rate. In stationary cells, the melanin produced accumulates within the cell and results in a rapid increase of melanin content per cell (accumulation effect). In growing cells, the melanin is diluted to daughter cells during division and results in a rapid decrease of melanin content per cell (division-dilution effect). If the melanin production rate equals the rate of dilution, the melanin content per cell will remain unchanged. In rapidly growing cells, if the dilution rate is greater than that of melanin production, melanin content per cell will decrease (Hu et al., 1995). Secondly, from the clinical viewpoint, pigmentation is a function of total melanin dispersed throughout the tissue, not of either the melanin content in a given melanocyte or the melanin production rate (Gordon et al., 1989). Therefore, melanin content per culture is used as the main parameter for evaluating the effect of various substances on melanogenesis of UM in vitro.

bFGF had a marked growth stimulating effect on cultured UM, followed by a secondary decrease of melanin content per cell (division-dilution effect). No significant difference of melanin content per culture has been observed in UM cultured with different levels of bFGF, indicating that bFGF may not affect melanogenesis in UM. bFGF activates various second messenger systems in different cells, but most probably stimulates the tyrosine kinase system in melanocytes (Halaban and Moellmann, 1990).

Previous reports on the effect of TPA on growth and melanogenesis in cultured EM and cutaneous melanoma cells have been conflicting (Huberman, Heckman and Langenbach, 1979; DeLuca et al., 1988; Herlyn et al., 1988; Bennett, 1989; Gordon et al., 1989; Brooks et al., 1990; Halaban and Moellmann, 1990; Halaban et al., 1993). Generally speaking, TPA stimulates the growth of EM and inhibits the growth of melanoma. TPA inhibits melanogenesis of murine melanoma cells and stimulates melanogenesis in human EM and cutaneous melanoma. In the present study, using melanin content per culture as the indicator, TPA stimulated

growth and melanogenesis in a dose-dependent manner. The effects of TPA on melanin content per cell are influenced by the stimulating effect of TPA on melanogenesis (the primary effect, which resulted in an increase of melanin content per cell) and by the secondary effect of growth stimulation (which resulted in a decrease of melanin content per cell). The increase of melanin content per cell by the melanogenesis stimulating effect of TPA is approximately equal to or slightly less than the decrease of melanin content per cell caused by the division-dilution effect secondary to growth stimulation. Therefore, the melanin content per cell slightly decreased or remains unchanged while the concentrations of TPA increased from 0.3 ng ml^{-1} to 300 ng ml^{-1}.

TPA is a tumor-promoting agent and a protein kinase C (PKC) activator. The effect of TPA on melanocytes has been attributed to its tumor-promoting properties (Huberman . et al., 1979). However, another phorbol ester which activates PKC but does not promote tumor formation (Sapintoxin A) also stimulates the growth of EM (Brooks et al., 1990). Furthermore, a PKC activating phorbol ester ($4-\beta$-phorbol didecanoate) stimulates the specific reaction of cultured EM, but an inactive isomer ($4-\alpha$-phorbol didecanoate) failed to stimulate growth of EM (Pittelkow and Shipley, 1989). These results indicate the effect of TPA on the melanocytes seems to depend on the activation of PKC (Pittelkow and Shipley, 1989; Brooks et al., 1990; Halaban and Moellmann, 1990).

In the present studies, three different cAMP-elevating agents were selected to represent three different mechanisms for increasing intracellular cAMP level. dbcAMP is capable of penetrating the cell membrane and raising the intracellular cAMP level directly. IBMX raises the intracellular cAMP level by inhibiting cAMP phosphodiesterase. CT raises the cAMP level by activating adenylate cyclase, which catalyses the synthesis of cAMP from intracellular ATP. All three substances produced a marked increase of melanin content per culture in cultured UM, indicating that elevating the intracellular cAMP level has the effect of stimulating melanogenesis in UM. This result is consistent with previous reports that cAMP-elevating agents stimulate melanogenesis of EM and cutaneous melanoma cells in vitro (Wade and Burkart, 1978; Abdel-Malek, 1987; Bennet, 1989; Halaban et al., 1993).

The effects of cAMP-elevating agents on the growth and melanogenesis of the UM parallelled one another. The melanogenesis stimulating effect of cAMP-elevating agents is dose-dependent within a defined range of concentrations. Melanin content per culture remains the same or slightly decreases when the concentration is higher than the optimal concentration.

In the present study, TPA and cAMP-elevating agents increased tyrosinase activity of UM, while bFGF

slightly decreased (20 ng ml^{-1}) or did not affect tyrosinase activity (1 ng ml^{-1}). These results paralleled their effects on melanin content per culture of UM in vitro.

The effects of bFGF, TPA and cAMP-elevating agents represent the effects of three important second messenger systems (tyrosine kinase system, PKC system and cAMP system) on melanogenesis in UM. Many growth factors, hormones, neurotransmitters and inflammatory mediators act on cells through these second messenger systems. Therefore, cultured UM could be used to study the effects and mechanisms of these factors on melanogenesis in UM. These studies are valuable for understanding the behavior of UM under physiologic and pathologic conditions in vivo. For example, studies on cultured UM may help to elucidate such phenomenon as the iris depigmentation seen in Horner's syndrome and Fuchs' heterochromic cyclitis and the increased pigmentation that has been observed in hypopigmented regions of some heterochromic irides of glaucomatous eyes after long-term treatment with a prostaglandin F2α analogue (Camras et al., 1994; Watson and Stjernschantz, 1994).

Acknowledgements

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Skin Organ Culture Model for Examining Epidermal Melanization

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To clarify the regulatory mechanism of skin pigmentation through epidermal proliferation and differentiation, organ cultures of pigmented guinea pig skins have been studied for their melanogenic responses to exogenous stimuli. Melanogenic activity was measured by both tyrosinase activity assessed by observing release of ^3H_2O from tissue after incubation with ^3H-tyrosine and melanin synthesis, indicated by the incorporation of ^{14}C-2-thiouracil into tissue. Those skin explants that were maintained in serum-free media under air conditions equilibrated with 5% CO_2, 50% O_2, and 45% N_2 formed new, chemically analyzable pigment in the tissue within 4 d of culture. This melanization was accompanied by an increased number of melanocytes as well as by enhanced tyrosinase activity and elevated melanin synthesis. This organ culture system responded well to known tyrosinase inhibitors such as phenylthiourea, which decreased melanogenic activity. Of the arachidonic acid metabolites tested, PGE$_2$, LTC$_4$, LTB$_4$, and 5-HETE were found to significantly stimulate melanogenic activity as indicated by tyrosinase activity, whereas PGF$_2$-alpha, 12-HETE, and 15-HETE did not. Among several known growth factors, only bFGF significantly stimulated melanogenesis in the organ cultured melanocytes. TGF-alpha, which increased DNA synthesis, had a slightly stimulating effect on melanogenesis, whereas TGF beta, which inhibited DNA synthesis, did not stimulate melanogenesis, but rather slightly decreased it. 8-methoxypsoralen + ultraviolet A–treated skin that underwent a marked pigmentation within 14 d in vivo demonstrated enhanced melanogenesis in the organ culture system in proportion to its in vivo pigmentation. Our organ culture system might provide an opportunity to examine the mechanism of action of epidermal melanization. J Invest Dermatol 100:47–54, 1993

Skin color is maintained by the constant presence of melanin in the epidermis. In the normal steady state, the mass of melanin within the epidermis is well regulated by a constant rate of production by melanocytes and by degradation by keratinocytes through the keratinization process. Melanin synthesis within melanocytes is genetically controlled by the activity of the key enzyme tyrosinase [1,2], which is generally thought to parallel the amount of melanin produced [3,4]. Upon stimulation such as ultraviolet (UV) irradiation [5–8] or allergic contact dermatitis [9], melanocytes increase in number, overproduce the melanin polymer, and transfer it through their dendrites to

surrounding epidermal cells, resulting in cutaneous hyperpigmentation. The mechanisms underlying the epidermal hyperpigmentation caused by several inflammations are thought to be associated primarily with the proliferation and differentiation of melanocytes [5–7]. However, little is known about the factors responsible for the hyperpigmentation that frequently occurs after UVB, 8-methoxypsoralen + UVA (PUVA), or UVA exposures [5,6]. Recently, studies have shown that keratinocytes make a large contribution to the proliferation or differentiation of melanocytes through mediators such as growth factors [10,11] or prostaglandins (PG) [12–14]. However, there is little evidence to suggest the mode of action of epidermal melanogenic stimulation because there is no suitable in vitro system to examine the process of hyperpigmentation in the epidermal unit. Such an in vitro system might provide an opportunity to examine the process of hyperpigmentation under conditions of experimental interest that would be impossible in vivo. We describe here an organ culture model that appears to maintain stimulated melanin formation that can be easily detected by skin-color change as well as by biochemical activities related to melanogenesis.

MATERIALS AND METHODS

Materials [^3H]dThd ([methyl-^3H]dThd, 2 Ci/mmol) and [^3H]tyrosine (1-[3,5-^3H]tyrosine, 40–60 ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{14}C] thiouracil ([2-^{14}C] thiouracil, 2 Ci/mmol) was obtained from Muromachi Chemical Co. (Tokyo, Japan). Hydrocortisone, 8-methoxypsoralen (8-MOP), phenylthiourea (PHTU), alpha-melanocyte–stimulating hormone (alpha-MSH), dibutyryl cyclic adenosine monophosphate (db-cAMP), and human epidermal growth factor were purchased from Sigma Chemical Co. (St. Louis, MO). PGE$_2$, PGF$_2$alpha,
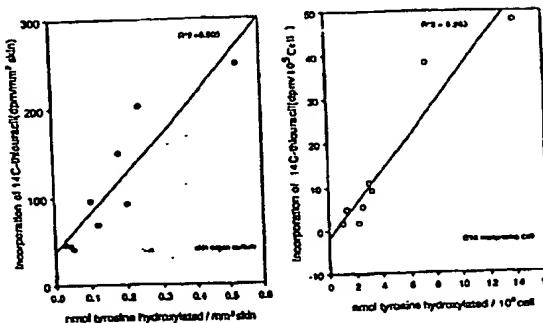
Figure 1. Correlation between nmol of hydroxylated tyrosine and incorporated ^{14}C-thiouracil in skin organ culture and B-16 melanoma cells. Assays were carried out for 4 d and 6 h, respectively, under several conditions of skin organ culture and B-16 melanoma cell culture.

leukotriene (LT)C_4, LTB$_4$, 5-HETE, 12-HETE, and 15-HETE were obtained from Cayman Chemical (Ann Arbor, Michigan). Human growth factors including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-alpha, and TGF-beta were purchased from Biomedical Technologies Inc. (Stoughton, MA). Human recombinant basic fibroblast growth factor (FGF) was obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). Human IL-1 and recombinant IL-6 were obtained from Wako Chemical Co. (Tokyo, Japan).

Preparation of Explants Organ cultures of guinea pig skin were established from a tortoiseshell guinea pig weighing 200 g. Flank skin was removed with a keratotome that excluded the hair bulb. Discs were taken from each skin sample with a 3-mm-diameter biopsy punch. The discs were washed in Eagle's minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) containing 50 μg/ml chloramphenicol, then put in a diffusion chamber composed of a milipore filter with a 0.22-μm pore size. This assembly was placed in a culture tube containing 2 ml of nutrient medium (see below). This tube was rotated at 37°C and 15 rpm under the air conditions equilibrated with 5% CO_2, 50% O_2, and 45% N_2.

PUVA Treatment The animals received 4.2 mg/kg body weight of 8-MOP in 30% ethanol intraperitoneally. Thirty minutes
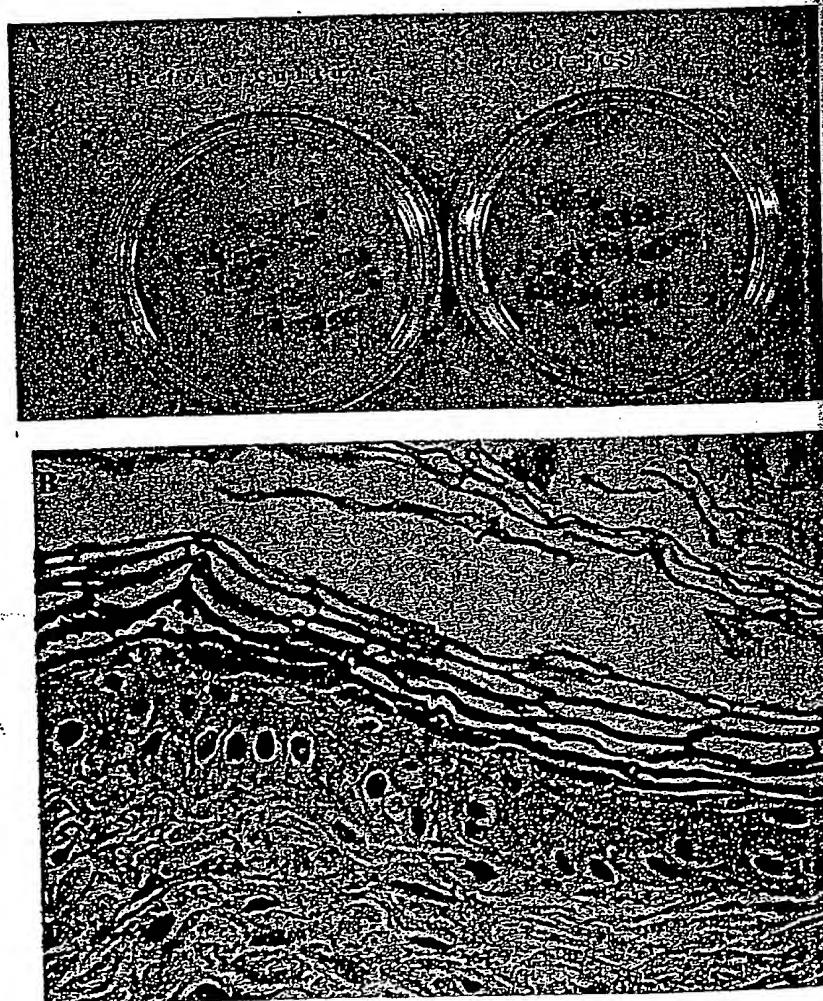

Figure 2. A) The appearance of pigmentation after 4 d of skin organ culture in comparison with non-culture. B) Hematoxylin and eosin section of organ cultured skin after 4 d.

after administration, they were exposed for 5 min to UVA of 3.1 mW/cm² at 365 nm (1 MPD = 3.5 j/cm²), emitted from Toshiba FL20BLB lamps. Degrees of pigmentation were evaluated according to the following scale: −, no reaction; ±, minimal visible pigmentation; +, moderate pigmentation; ++, intense, deep pigmentation.

Nutrient Medium   The nutrient medium was prepared from Eagle's MEM containing 4 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 20 mM Hepes (pH 7.3). In addition, the medium was supplemented with hydrocortisone (0.4 µg/ml) and epidermal growth factor (1 ng/ml).

Tyrosinase Activity   Tyrosinase activity was assayed according to the method of Oikawa et al [15]. Briefly, ³H-tyrosine was added to the medium at a concentration of 1 µCi/ml throughout the period of organ culture. After culture, 200 µl of medium was assayed for release of ³H₂O, after removing non-reacted ³H-tyrosine through three cycles of absorption into activated charcoal. The quantity of hydroxylated tyrosine was calculated from the ratio of the released ³H₂O (dpm) to the added ³H-tyrosine (dpm) for the total amount of tyrosine present in the medium. In addition, the incorporation of ¹⁴C-thiouracil into skin tissue was measured as another indicator of tyrosinase activity [16-18]. Thus, 0.5 µCi of ¹⁴C-thiouracil was added to the medium throughout organ culture and the amount incorporated was measured after washing with an excess of unlabeled thiouracil in phosphate buffer (0.1 M, pH 7.4) for 24 h, followed by three washes with 5% trichloroacetic acid (TCA), solubilization of skin tissue with 2 N NaOH, and neutralization with 2 N HCl.

Histochemical Procedures   Discs of skin were rinsed in 0.1 M phosphate buffer (ph 6.8) and incubated in 1 M sodium bromide for 5 h at 37°C. The epidermal sheets separated from the dermis were fixed in 10% cold neutral formalin for 30 min, washed twice with 0.1 M phosphate buffer (pH 6.8), and incubated in 0.1% dihydroxy-phenylalanin (Dopa) in 0.1 M phosphate buffer (pH 6.8) for 5 h. In some cases, the epidermal sheets were further stained by the Fontana-Masson silver method. The number of melanocytes per square milimeter was counted using an Olympus-BHA microscope
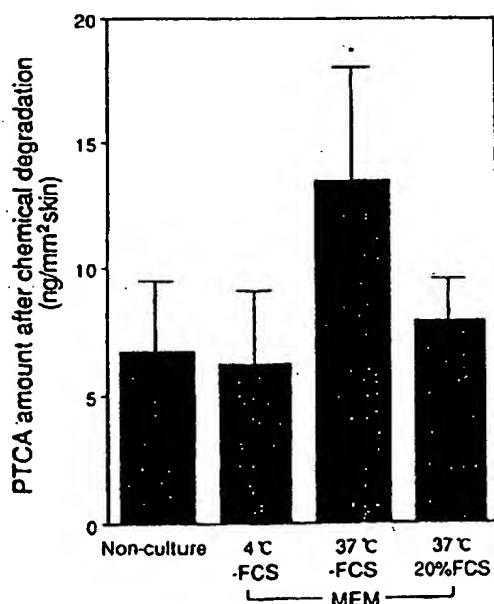

Figure 3. Chemical analysis of eumelanin (PTCA) after 4 d of skin organ culture. The data are expressed as mean ± SD of triplicate determinations. * p < 0.05.
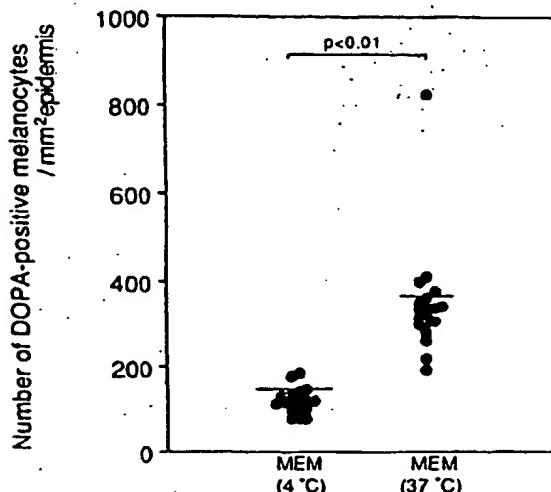

Figure 4. The increase in Dopa-positive melanocytes after 3 d of organ culture.

at a magnification of ×200. In each specimen, the number of melanocytes was calculated by averaging the numbers found in 50 – 100 fields.

Quantitative Analysis of Melanin   Melanin amounts were analyzed by Ito's method [19] for eumelanin (PTCA) and expressed as ng/mg wet skin tissue.

Incorporation of ³H-TdR   DNA synthesis in skin slices was assayed by measuring the incorporation of ³H-TdR. ³H-TdR (1 µCi/ml) was added to the organ culture for the indicated periods. After incubation, the skin slices were washed three times with cold Hanks' balanced solution, and 5% cold TCA was added twice every 10 min. The TCA-insoluble tissue was dissolved in 2 N NaOH and neutralized with 2 N HCl. The radioactivity was counted in a liquid-scintillation spectrometer.

RESULTS

To confirm the reliability of the incorporation of ¹⁴C-thiouracil as an indicator for melanin synthesis, we compared the extent of its incorporation with the amount of tyrosine hydroxylated (calculated from the release of ³H₂O) at various stages of organ culture. The incorporation of ¹⁴C-thiouracil was found to occur in parallel with the hydroxylation of tyrosine, with a relation coefficient of 0.905 (Fig 1). This indicated that the incorporation of ¹⁴C-thiouracil is a useful indicator of melanin synthesis.

Skin explants maintained in a serum-free medium containing hydrocortisone and EGF showed newly formed pigment within 4 d of culture (Fig 2). Chemical analysis of melanin (Fig 3) demonstrated that there was about a twofold increase in eumelanin (PTCA) in cultured tissue as compared to the level before culture. The stimulating effect on melanin synthesis disappeared in the presence of 20% fetal calf serum (FCS). Dopa and Fontana-Masson silver histochemistry of split epidermis showed that Dopa-positive melanocytes increased in number twofold during the period of organ culture (Fig 4), the melanocytes had a round appearance, and much melanin pigment was transferred to keratinocytes (Fig 5). DNA synthesis, as demonstrated by thymidine incorporation into organ cultured skin, continued to occur until at least day 7 of culture. Melanin synthesis as measured by ¹⁴C-thiouracil incorporation appeared to proceed for 7 d of culture, parallel to DNA synthesis (Fig 6). To examine the inhibitory effect of tyrosinase inhibitors on this melanogenic stimulation, phenylthiourea was tested at 0.1 mM concentrations and was found to exhibit a significant inhibitory effect on melanogenesis, as measured by both the quantity of tyro-
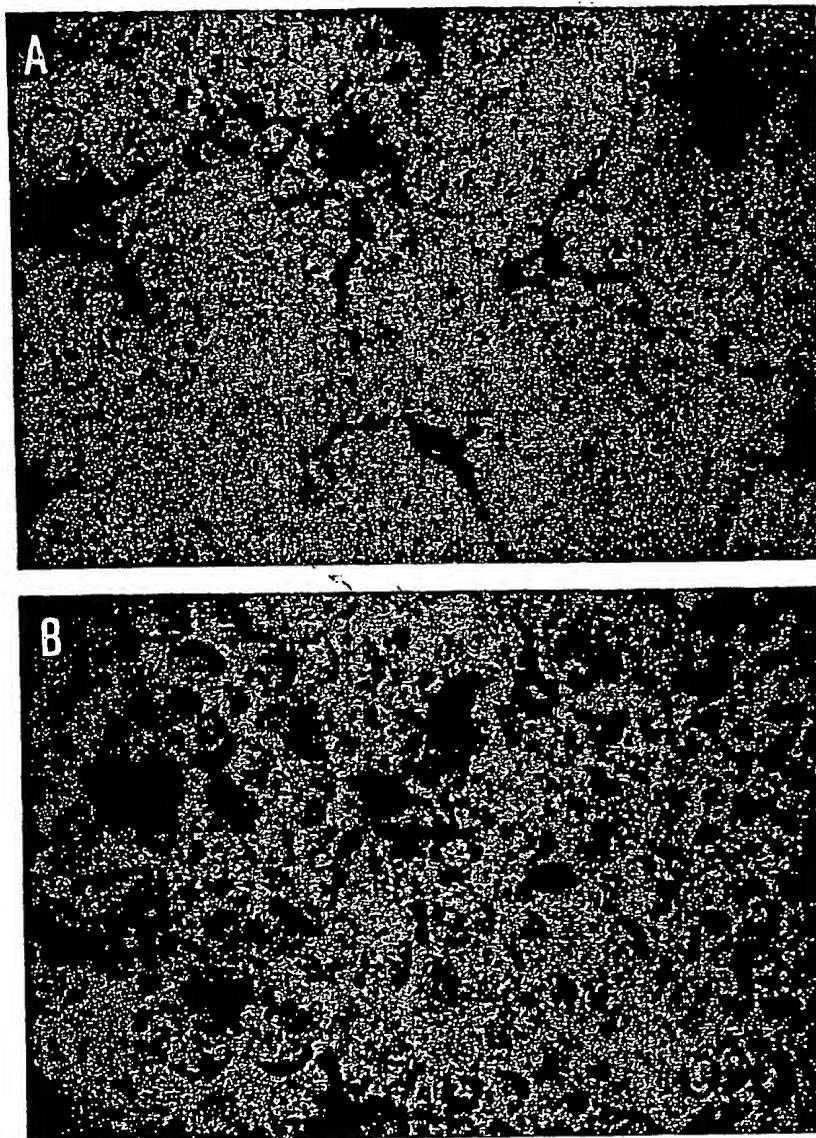
Figure 5. Dopa and Fontana-Masson silver histochemistry of split epidermis after 3 d of organ culture. A) 0-d culture. B) 3-d culture.

sine hydroxylated and the extent of incorporation of ^{14}C-thiouracil (Fig 7).

We evaluated the stimulatory effects of exogenous materials and found that of arachidonic acid metabolites, including prostaglandins and leucotrienes, PGE$_2$, at a concentration of 1.0 mM, significantly stimulated melanogenic activity in comparison with control culture as indicated by tyrosine hydroxylation or thiouracil incorporation (Fig 8). Furthermore, the addition of LTC$_4$, LTB$_4$, and 5-HETE significantly increased melanogenic activity as measured by both melanogenic assays, whereas PGF$_2$-alpha, 12-HETE, and 15-HETE did not show any stimulatory effect (Fig 9). Among several known growth factors, such as bFGF, PDGF, interleukin (IL)-1, and IL-6, only bFGF stimulated melanogenesis in the organ cultured melanocytes, but the addition of db-cAMP abrogated this stimulatory effect (Fig 10). Under these organ culture conditions, TGF-alpha and TGF-beta were effective in significantly increasing

or decreasing DNA synthesis, respectively (Fig 11). Under the same organ culture conditions, TGF-alpha was found to increase slightly tyrosinase activity at an optimal concentration of 10 ng/ml, whereas TGF-beta did not stimulate melanogenesis, but rather decreased it (Fig 12). When PUVA-treated skin was cultured several days post-exposure, melanin synthesis as indicated by thiouracil incorporation was significantly stimulated, corresponding to the degree of in vivo pigmentation (Fig 13). The stimulated melanogenesis was further significantly enhanced or inhibited by additions of PGE$_2$ and PHTU, respectively.

DISCUSSION

Melanocytes are cells that produce and secrete melanin granules into keratinocytes, thereby controlling the quantity of melanin within the epidermis. Control mechanisms of integumental melanization have been a key interest of many investigators; however, the mecha-
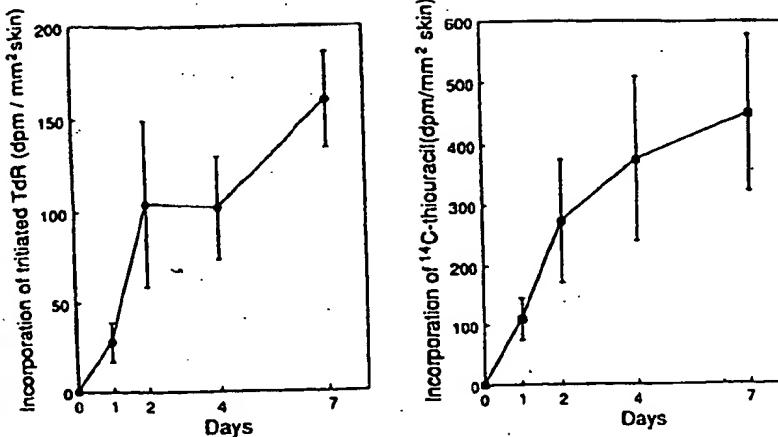
Figure 6. DNA synthesis and ^{14}C-thiouracil incorporation during 7 d of organ culture. Cumulatively incorporated radioisotopes were counted in the indicated days after the onset of skin organ culture. The data are expressed as mean ± SD of triplicate determinations.

nism of hyperpigmentation that frequently occurs after UVB or UVA irradiation remains unclear, as do phototoxic reactions, for example, to PUVA treatment [5,7]. Research on the mechanism of hyperpigmentation has focused on the behavior of cultured melanocytes in the presence of several stimulants. This mechanism has been ascribed primarily to the proliferation of melanocytes [8] and their concomitant or subsequent differentiation, induced by eicosanoids released from keratinocytes during the process of inflammation [12,13].

The present study demonstrated that organ cultured skin is capable of continuing or stimulating melanin production, accompanied by an increased number of melanocytes and enhanced tyrosinase activity. This melanogenesis was significantly enhanced in the organ culture of PUVA-treated skin, corresponding to the time course of in vivo pigmentation. All melanogenic stimulation was suppressed by the concomitant addition of the specific tyrosinase inhibitor, PHTU [20]. The addition of FCS markedly inhibited the stimulated melanization, and eventually resulted in normal levels of

melanin in the skin. This observation suggests that serum plays an important role in maintaining the normal level of melanization, either through the actions of tyrosinase inhibitors in serum [21] or by unknown mechanisms. Thus, the melanogenic responses of organ cultured skin indicate that, because of the long period of maintenance of skin tissue, the present organ culture system is a reliable epidermal unit for evaluation of the melanogenic control mechanisms underlying skin pigmentation homeostasis, as well as hyper- or hypopigmentation processes.

Recently, several endogenous soluble factors have been implicated in UV-mediated hyperpigmentation as well as in the normal maintenance of skin color [10,11]. PGE_2 has been shown to be an important endogenous stimulant in UV-induced post-inflammatory hyperpigmentation [12,13]. There have been many reports describing the role of PGE_2 in the stimulation of melanogenesis. In those studies, the addition of PGE_2 into several in vivo or in vitro systems was found to stimulate melanogenic activity [12,14]. An important feature of the organ culture system discussed here is that, of the arachidonic metabolites tested, the addition of PGE_2 produces
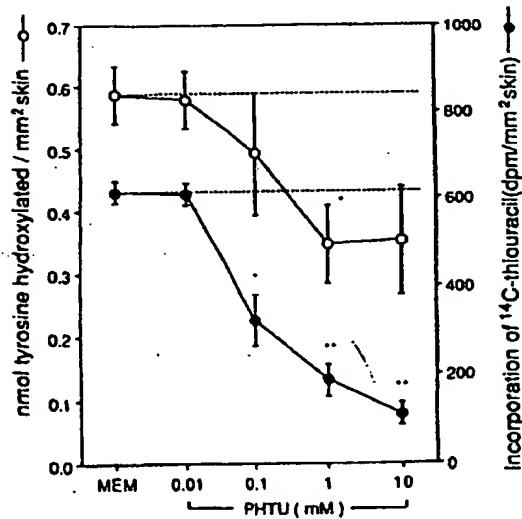
Figure 7. Inhibitory effect of PHTU on the melanogenesis of organ cultured skin as measured by nmol of tyrosine hydroxylated and ^{14}C-thiouracil incorporation over 4 d of culture. The data are expressed as mean ± SD of triplicate determinations. **p < 0.01; *p < 0.05 as compared to MEM (medium control).
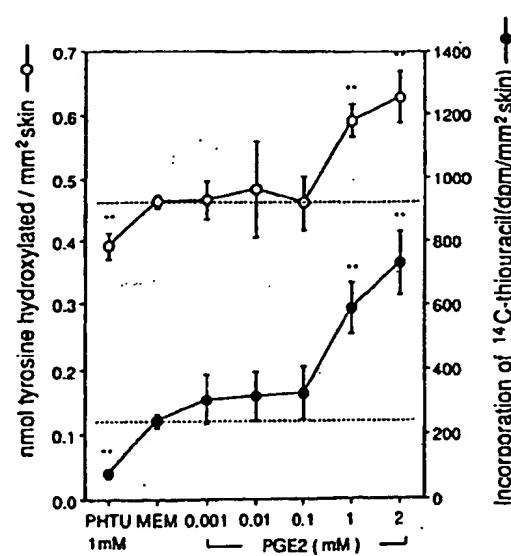
Figure 8. Melanogenic stimulation by PGE_2 in organ cultured skin as measured by nmol of tyrosine hydroxylated and ^{14}C-thiouracil incorporation over 4 d of culture. The data are expressed as mean ± SD of triplicate determinations. **p < 0.01 as compared to MEM.
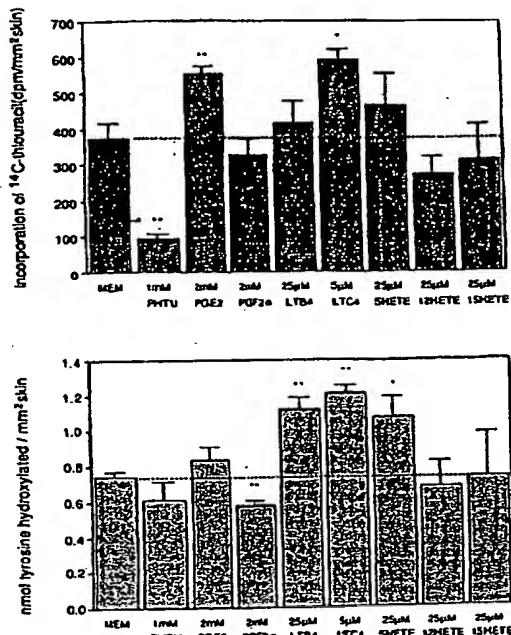
Figure 9. Melanogenic stimulatory effect of lipoxygenase metabolites in organ cultured skin as measured by nmol of tyrosine hydroxylated and ^{14}C-thiouracil incorporation over 4 d of culture. The data are expressed as mean ± SD of triplicate determinations. **$p < 0.01$; *$p < 0.05$ as compared to MEM.

a marked stimulatory effect on melanin synthesis, as revealed by increased incorporation of thiouracil as well as by the release of ^3H_2O. However, this stimulation is observed at much higher concentrations than those under physiologic skin conditions. The requirement for high PGE_2 concentrations to elicit melanogenic activation may be because, in this organ culture system, it is difficult for exogenous PGE_2 to reach melanocytes, due to the abundance of surrounding cells with prostaglandin receptors that interrupt the passage of prostaglandins. Because PGE_2 is synthesized mainly in keratinocytes under normal or stimulated skin conditions, keratinocytes can be considered an essential control unit for the melanogenesis of melanocytes in epidermis. As well as PGE_2, lipoxygenase products such as LTB_2, LTC_4, and 5-HETE were also found to stimulate epidermal melanogenesis in our organ culture system. This is in agreement with a recent report that LTC_4 is a mitogen for human melanocytes even in the absence of TPA [22].

Another mechanism of keratinocytes for controlling melanogenesis is suggested to be through the secretion of growth factors. Halaban et al [10] reported that human melanocytes are considerably stimulated to proliferate in the absence of phorbol ester by cell extracts from proliferating keratinocytes. This effect is abolished by concomitant addition of antibody against bFGF. In contrast, Gordon et al [11] demonstrated no role for bFGF in melanocyte proliferation by showing no neutralization of the conditioned medium produced by keratinocytes by anti-bFGF antibody. Our experiment with bFGF demonstrated that bFGF can act on organ cultured melanocytes to stimulate melanogenesis, thus suggesting an important role for bFGF in the proliferation as well as in the melanogenesis of melanocytes. In contrast to Halaban's results using pure melanocyte cultures [10], the addition of db-cAMP cancelled the stimulating effect, indicating that db-cAMP may also produce cellular reaction in keratinocytes that abrogate the action of bFGF on melanocytes.

Physiologic melanogenic stimuli such as sun exposure are known to induce the secretion of IL-1 [23,24] and IL-6 [25], and to increase

A

B
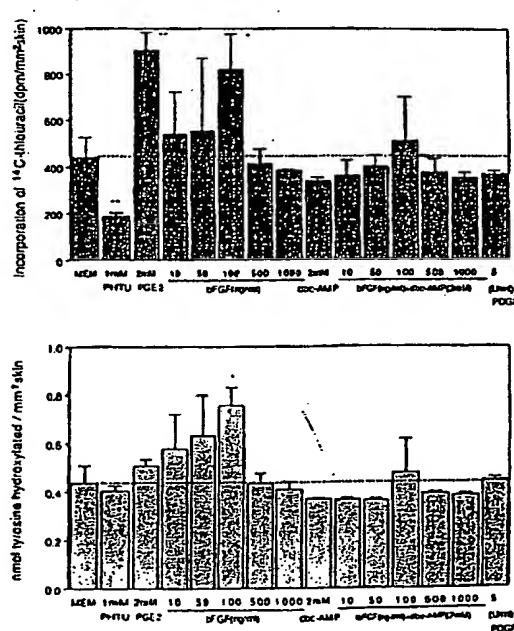
Figure 10. Melanogenic stimulatory effect of several growth factors including IL-1, IL-6, PDGF, and bFGF in organ cultured skin as indicated by nmol of hydroxylated tyrosine and ^{14}C-thiouracil incorporation over 4 d of culture. A) The effects of bFGF and PDGF. B) The effects of IL-1 and IL-6. The data are expressed as mean ± SD of triplicate determinations. **$p < 0.01$; *$p < 0.05$ as compared to MEM.
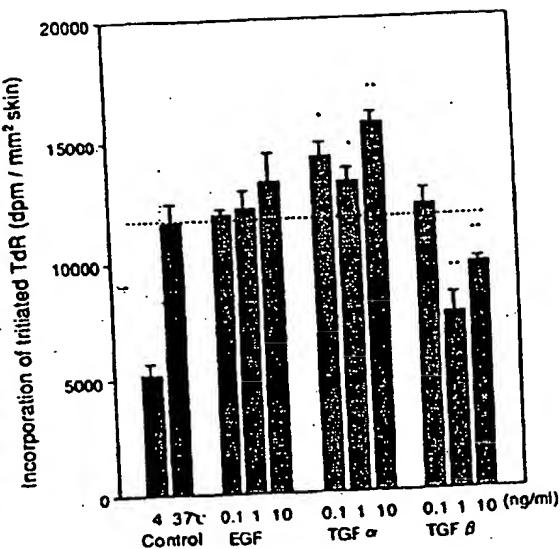
Figure 11. Effects of transforming growth factors on DNA synthesis of organ cultured skin. In this experiment, EGF was eliminated from the organ culture medium. The data are expressed as mean ± SD of four determinations. **p < 0.01; *p < 0.05 as compared to control.

the proliferation of epidermal cells. Rapidly proliferating keratino-cytes are found to produce higher levels of growth factors such as bFGF [10] and TGF alpha [26,27] than do stratifying keratinocytes. Highly pigmenting melanocytes are often located in close proxim-ity to rapidly dividing epidermal cells, as seen in heavily pigmented deep rete ridges and anagen hair follicles, leading to the suggestion that highly proliferating keratinocytes secrete growth factors that
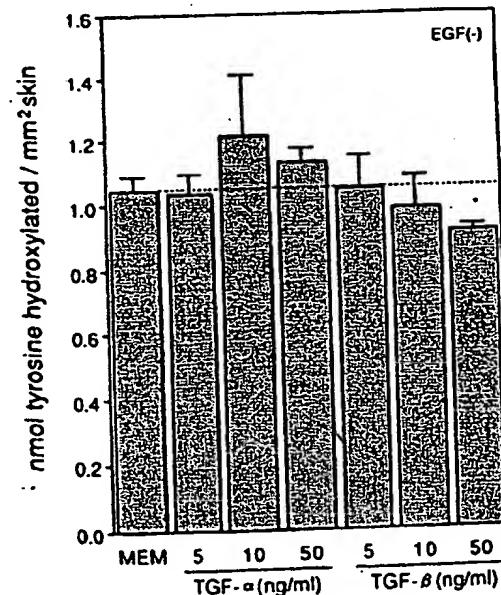

Figure 12. Stimulating effects of TGF-alpha on the melanogenesis of organ cultured skin as measured by nmol of hydroxylated tyrosine and [14]C-thiouracil incorporation over 4 d of culture. In this experiment, EGF was eliminated from the organ culture medium. The data are expressed as mean ± SD of triplicate determinations. *p < 0.05 as compared to MEM.
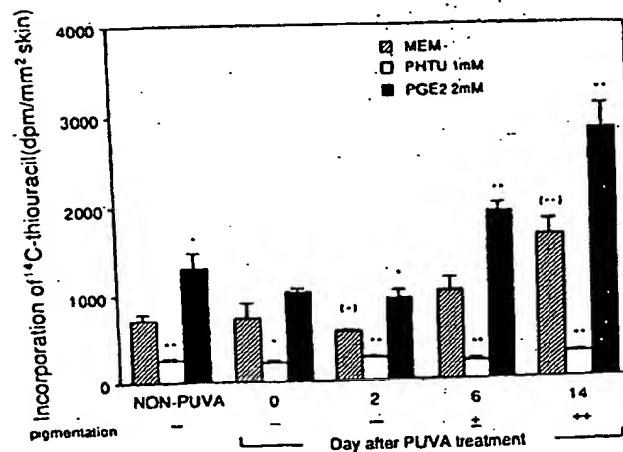

Figure 13. Melanogenic stimulation in the organ culture of PUVA-treated skin corresponding to their in vivo pigmentation process. The data are ex-pressed as mean ± SD of triplicate determinations. **p < 0.01; *p < 0.05 as compared to MEM of NON-PUVA: **p < 0.01; *p < 0.05 as compared to corresponding MEM.

stimulate melanocytes in a paracrinic manner. Our results using TGF-alpha and TGF-beta suggest that epidermal growth stimula-tion and inhibition influence epidermal melanization. However, the precise mechanism underlying the stimulation or inhibition of melanogenesis by epidermal growth factors will remain unclear until a time-course study using a shorter assay time has been per-formed.

In conclusion, the data presented here demonstrate the role of the epidermal unit in cutaneous pigmentation through the recognition of melanogenic responses to several intrinsic factors in organ cul-tured skin. Ongoing studies to characterize the behavior of melano-cytes within the epidermal unit should provide a better understand-ing of the regulatory mechanisms of epidermal melanization.

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Tyrosinases from Two Different Loci Are Expressed by Normal and by Transformed Melanocytes*

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Two pigmentation related genes have recently been cloned which map to the *brown* (*b*) and *albino* (*c*) loci of mice; these loci influence the quality and quantity, respectively, of melanin produced by melanocytes. Both these gene products are biochemically similar and have extensive amino acid sequence similarity to each other and to lower forms of tyrosinase (EC 1.14.18.1), a copper binding enzyme responsible for melanin production. In order to characterize the catalytic activities of these molecules, we have synthesized peptides and prepared antibodies to them which specifically recognize the gene products in question. By use of immune affinity purification protocols, we have isolated the proteins encoded by the *brown* and *albino* loci and have determined that both have the catalytic functions ascribed to tyrosinase, *i.e.* hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPAquinone. These are the critical reactions to melanogenesis since melanin pigment can be spontaneously produced from those products. The specific activity of the *albino* locus encoded product is considerably higher than that of the protein encoded by the *brown* locus, although the latter protein is present in higher quantity in melanocytes than is the protein encoded by the *albino* locus. These results are surprising since it was anticipated that tyrosinase was the product of single gene locus, and suggest that regulation of melanogenesis in mammals is controlled at the enzymatic level by several different gene products.

Tyrosinase (EC 1.14.18.1) is a bi- (and possibly tri-) functional enzyme critical to the formation of melanin in mammals and lower animals (*cf.* Refs. 1–3 for recent reviews). In a complex series of reactions as yet incompletely defined, melanin is produced as a highly polymerized complex of products resulting from: (*a*) the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA),[1] (*b*) the oxidation of DOPA to DOPAquinone, (*c*) the internal cyclization of DOPAquinone to indole-quinone, and (*d*) the further oxidation and polymerization of melanogenic intermediates derived

from these metabolites. Although tyrosinase is the only enzyme known to be essential to this pathway (since DOPA can spontaneously autooxidize and the remainder of the chemical reactions leading to melanin production can proceed noncatalytically), several factors have been described which follow the action of tyrosinase and can influence the production of melanin in mammals either in a positive or a negative fashion. It has been recently shown that DOPAchrome isomerase (4–8), peroxidases (9) and/or catalases (10), glutamine metabolic enzymes (11, 12), metal cations (13, 14), and melanogenic inhibitors (15, 16) all potentially function in the regulation of melanogenesis.

Pigmentation in mammals occurs from the deposition of melanin produced by melanocytes in the skin, hair bulbs, and eyes. Melanogenesis can be influenced at many levels. In the mouse, for example, more than 150 different mutations have been defined at more than 50 distinct genetic loci that affect the production and distribution of melanin. Several of these genetic loci directly affect the expression of tyrosinase activity, and thus melanin formation, although the structure and function of the molecules encoded by those loci have not yet been characterized. For example, the *A^y* (*lethal yellow*) and the *e* (*extension*) loci affect the type of melanin produced (black eumelanin *versus* yellow sulfhydryl-containing pheomelanin), while the *b* (*brown*), *p* (*pink*), and *c* (*albino*) loci affect the level of demonstrable tyrosinase in melanocytes. Which of these loci, if any, encodes tyrosinase and which are regulatory has been disputed for decades (*cf.* Refs. 17–20 for reviews). It has generally been assumed that tyrosinase is the product of a single genetic locus, and that the various isozymic forms demonstrable for tyrosinase result from post-translational modification of a single molecule (1–3, 21–23). In view of the lack of melanin production by *albino* mutants, and because of other genetic considerations, the *albino* locus has often been postulated, or assumed to be, the structural locus for tyrosinase (24–26). However, this notion has not been universally accepted in light of biochemical evidence which demonstrates the presence of active tyrosinase in many *albino* mutations, and from the many other pleiotropic effects that result from mutations at the *albino* locus (27–30).

Several laboratories have recently cloned genes which map to either of two of the relevant loci noted above (the *albino* and *brown* loci in mice) (31–43); the deduced amino acid sequences encoded by those genes are highly similar (20, 32, 36), possess most of the characteristics expected for tyrosinase, and bear striking identity to lower forms of tyrosinase (recently reviewed in Ref. 20). In attempting to define the biochemical and functional relationships of the proteins encoded by the *brown* and *albino* loci, and the mechanisms by which they interact and influence melanin production, we have prepared antibodies to synthetic peptides and have examined the expression and catalytic activities of those gene products. In a previous study (44), we demonstrated that the

[1] The abbreviations used are: DOPA, 3,4-dihydroxyphenlalanine; ELISA, enzyme-linked immunosorbent assay; MSH, melanotropin (α-melanocyte-stimulating hormone); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

protein encoded by the *brown* locus (also referred to as the *b*-protein) has both of the catalytic properties ascribed to tyrosinase, that is, tyrosine hydroxylase and DOPA oxidase activities. We now demonstrate that the protein encoded by the *albino* locus (alternatively termed the *c*-protein) is also competent for both tyrosinase catalytic activities, and in fact has an even higher specific catalytic activity than the *b*-protein. The *b*-protein is preferentially expressed by transformed melanocytes while the *c*-protein is preferentially expressed by normal melanocytes *in vivo*; a protein which results from alternative mRNA processing of the *albino* locus transcript (33, 45) is expressed by both types of melanocytes. In addition, yet another tyrosinase-related protein, termed TRP2, has recently been cloned.[2] These results, taken in context with the identification of other distinct but homologous tyrosinase-related genes, support the concept that melanogenesis in mammalian melanocytes is regulated by a family of distinct tyrosinase-related genes. Although the exact roles the *b*- and *c*-proteins play in the regulation of melanin production and how they interact with each other and these other melanogenic molecules are not yet clear, it is obvious from this report and from studies underway in many laboratories that a reassessment of the enzymatic controls involved in the regulation of mammalian pigmentation is required.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—JB/MS and B16 murine melanoma cells were routinely cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, glutamine, and antibiotics, as previously detailed (44, 46); all tissue culture products were obtained from GIBCO. MNT1 human melanoma cells were a gift of Dr. Pier Giorgio Natali, Regina Elena Cancer Institute, Rome, Italy. Murine and human "normal" melanocytes growing in culture were kindly supplied by Drs. Zalfa Abdel-Malek and Raymond Boissy (DBA and NHM) of the Department of Dermatology, University of Cincinnati, Cincinnati, OH; by Dr. Ruth Halaban (B10.BR), Department of Dermatology, Yale University School of Medicine, New Haven, CT; and by Dr. Dorothy Bennett (melan-a), Department of Anatomy, St. George's Hospital Medical School, London, United Kingdom; they were grown as detailed by those laboratories (47–49). Normal human melanocytes were also obtained from Clonetics Corp. (San Diego, CA) and cultured as per instruction. When cells were treated with α-melanocyte stimulating hormone (MSH, Calbiochem), they were grown in the presence of 2×10^{-7} M MSH for 4 days, conditions under which optimal stimulation of melanogenesis occurs (1, 46, 50). All cell cultures were routinely harvested with trypsin and washed twice in Hanks' balanced salt solution prior to solubilization or subculturing. Melanocytes were also obtained from *in vivo* sources, including transformed melanocytes from B16 and JB/MS melanomas (growing as solid pigmented subcutaneous tumors) and normal melanocytes (from the skin and eyes) of 5-day newborn pathogen-free C57BL/6N mice obtained from the Small Animal Section at NIH.

Peptide Synthesis—Peptides representing the carboxyl and amino termini of putative tyrosinase molecules were prepared by solid phase synthesis; peptides 1 and 2 were synthesized by Dr. W. Lee Maloy of the Biological Resources Branch at NIH (44), peptides 5 and 6 were synthesized by the Yale University Medical School Synthetic Peptide Service (New Haven, CT), and peptide 7 was synthesized by OCS Laboratories (Denton, TX). The purity of the peptides was established by amino acid analysis, and following purification by reversed phase high performance liquid chromatography, aliquots of the peptides were coupled to keyhole limpet hemocyanin as previously detailed (44, 46) and used for immunization of rabbits.

Antibody Production—Polyclonal antibodies to peptides were produced in New Zealand White rabbits by immunization and boosting with conjugated peptides as reported in earlier studies (44, 46); production of antibodies was quantified by an enzyme-linked immunosorbent assay (ELISA), and when high titers of specific antibodies had been raised, IgG fractions from the sera were purified using protein A-Sepharose columns (Pierce Chemical Co.). The TMH-1

monoclonal antibody is an IgG$_{2A}$ specific for the T$_4$ high M_r form of tyrosinase (51).

Metabolic Labeling and Immunoprecipitation—For metabolic labeling, cells were grown to semiconfluence, and then pulsed for 30 min with [^{35}S]methionine (Du Pont-New England Nuclear) and chased for 60 min in complete medium, as previously detailed (44, 46). Following harvesting, the cells were solubilized in 1% Nonidet P-40 in 10 mM Tris-HCl, pH 7.2, for 1 h at 4 °C. 5×10^6 dpm of [^{35}S]methionine-labeled extracts were incubated with antibodies as noted in the figure legends for 60 min at 37 °C, and then complexed with 100 μl of 10% protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) for 30 min. The immune complexes were washed 5 times with 0.1% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 7.2, and eluted with Laemmli SDS sample buffer for 5 min at 100 °C. When extracts were denatured prior to immunoprecipitation, they were treated with 1% SDS and 2 mM dithiothreitol at 70 °C for 5 min, and then diluted with 5 volumes of Nonidet P-40/Tris buffer containing 20 mM iodoacetic acid, and reacted with antibodies as noted above.

Immunodepletion experiments were performed by sequentially treating solubilized extracts with antibodies as described above, using the supernatants from each reaction for each subsequent immunoprecipitation. After 3 cycles of such absorptions, metabolically labeled proteins remaining in the supernatants were subjected to a final antibody absorption (as noted in the figure legend) and immunoprecipitated antigens were analyzed as noted above.

For further studies on the specificity of these antibodies, ^{35}S-labeled proteins immunoprecipitated by the peptide antibodies were separated by gel electrophoresis and the positions of the immunoprecipitated labeled bands were identified on identical gels autoradiographed overnight. The bands were then sliced out of the gel, minced with razor blades, and extracted into buffer overnight at 4 °C. Labeled proteins purified in this manner were then further analyzed for reactivity with antibodies, by isoelectric focusing, and for partial proteolytic digestion with *Staphylococcus aureus* protease (Pierce Chemical Co.).

Electrophoretic Techniques—The Laemmli SDS-gel electrophoresis system was routinely used with 7.5% acrylamide gels (52), although 10% gels were used in the partial proteolytic degradation experiments. When labeled proteins were resolved, the gels were fixed in 10% trichloroacetic acid, then treated with Fluoro-^{3}Hance (Research Products International, Mount Prospect, IL) for 30 min, dried, and autoradiographed on XAR-2 film with Cronex-II intensifying screens. M_r were estimated by comparison with the mobilities of methylated ^{14}C-labeled standard proteins (Du Pont-New England Nuclear).

For Western blotting, proteins from Nonidet P-40 extracts were separated by SDS-gel electrophoresis (with or without prior denaturation with mercaptoethanol and heat), then transferred to nitrocellulose papers in 25 mM phosphate buffer, pH 6.5, at 15 V overnight at 23 °C, as previously reported (44, 46). Following transfer, the sheets were stained with 0.01% Fast green to visualize and mark standard proteins and then were incubated in 1% bovine serum albumin in Nonidet P-40/phosphate-buffered saline (PBS) overnight at 4 °C to destain the filters and to block nonspecific binding. The blots were then incubated with antibodies (diluted at 1:100) as noted in the figure legends for 60 min at 37 °C and washed five times in Nonidet P-40/PBS; they were then incubated with the appropriate peroxidase-conjugated second antibody (DAKO Corporation, Santa Barbara, CA) (diluted at 1:500 in Nonidet P-40/PBS) for 30 min at 37 °C. The sheets were again washed thoroughly and specifically bound antibodies were visualized (A_{450}) by reaction with 3,3'-diaminobenzidine (2.5 μg/ml) and H$_2$O$_2$ (0.01%) in PBS. For Western blot analysis of immune-affinity purified proteins, the alkaline phosphatase biotin-avidin ABC staining kit was used according to the manufacturer's instructions (Pierce Chemical Co.).

Isoelectric focusing techniques were performed as previously detailed (2); ampholines were obtained from Pharmacia LKB Biotechnology Inc.

Immune-affinity Chromatography—Proteins were purified by immune affinity using two different protocols. In the first, IgG from 1 ml of the rabbit sera were bound directly to 1 ml of protein A-Sepharose columns (Pierce Chemical Co.) and the columns were washed extensively in buffer as per the manufacturer's instructions. In the second protocol, 1 ml of protein A-Sepharose columns were used to prepare purified IgG from the sera of immunized rabbits following the manufacturer's instructions. Four mg of these purified IgG were then covalently linked to immunoPure antibody immobilization columns (Pierce Chemical Co.), again following the manufac-

[2] I. J. Jackson, personal communication.

turer's instructions, and the columns were washed extensively in buffer prior to use. In both protocols, 1-ml aliquots of an Nonidet P-40 extract of B16 melanoma cells (~1 mg of protein) were applied to each column and allowed to bind for 1 h at 23 °C; following the binding, unbound proteins were removed by extensive washing with equilibration buffer. Specifically bound proteins were then removed sequentially with 1% SDS in equilibration buffer, or with the Elution buffer supplied with the columns. All fractions were collected, dialyzed extensively against 0.1% Nonidet P-40 in 10 mM Tris-HCl, pH 7.2, concentrated to 1 ml with Centricon 30 miniconcentrators, and used for immune reactivity studies employing Western immunoblot techniques as described above, or assayed for melanogenic activities as detailed below.

Melanogenic Assays—The various assays for melanogenic activities were performed as previously detailed (53, 54). Briefly, the [^{3}H] tyrosine assay specifically measures tyrosine hydroxylase activity; this is the initial reaction in the conversion of tyrosine to melanin, and involves the hydroxylation of tyrosine to DOPA. The formation of ^{3}H-water specifically and stoichiometrically released in this reaction is quantified using liquid scintillation counting. The DOPA oxidase assay measures the second major catalytic reaction, i.e. the conversion of DOPA to DOPAchrome (via DOPAquinone). This reaction is followed spectrophotometrically by the chromogenic appearance of DOPAchrome (A_{475}). The DOPAchrome isomerase assay is also a spectrophotometric assay which measures the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid or to dihydroxyindole. This reaction is also followed spectrophotometrically as the disappearance of DOPAchrome (A_{475}); this activity is thought to be catalyzed independently of tyrosinase (4–8). Finally, the [^{14}C] melanin assay measures the production of acid-insoluble melanin from the labeled tyrosine precursor and quantifies the entire reaction sequence, including tyrosinase and any post-tyrosinase catalytic activities. The production of the acid-insoluble [^{14}C]melanin is measured using liquid scintillation counting.

RESULTS

Preparation and Specificity of Anti-peptide Immunoglobulins—Synthetic peptides used in this study which correspond to the amino and carboxyl termini of the proteins encoded by the murine *brown* (b) locus and by the *albino* (c) locus are listed in Table I. High titers of antibodies were raised against all these peptides; the antibodies were extremely peptide-specific and could be competed for by the immunizing peptide, but not by other peptides. In addition, none of these antibody probes reacted with any of the other synthetic peptides bound to ELISA plates, further confirming their specificity. Note

especially that αPEP1 and αPEP7 antisera gave virtually identical titers of activity against the immunizing peptides, and that those activities were completely peptide-specific.

It should be noted that the PEP5 sequence represents the amino terminus of *all* the proteins produced from the murine *albino* (c) locus that have been identified to date, including those encoded by Tyrs-33 (33), MTY811 (35), Ty2 (39), tyr1 (40), Tyrs-J (41), and pmcTyr1, pmcTyr2, and pmcTyr3 (45). On the other hand, the PEP7 sequence represents the carboxyl terminus of those same proteins, with the exception of that encoded by Tyrs-33, whose unique carboxyl terminus is that noted as PEP6. The deletions in primary sequence of pmcTyr2 and pmcTyr3, and presumably of Tyrs-33, have been shown to be generated due to alternative splicing of the primary transcript (40, 45) and to result in the production of proteins that are catalytically inactive, or at least altered in function (36, 41). PEP1 and PEP2 represent the carboxyl and amino termini of the protein encoded by pMT4 (31); although only a single protein species has thus far been identified from the murine *brown* locus, it is reasonable to expect that related products generated by alternative splicing might also exist, since this gene has also been shown to consist of multiple introns and exons (55), as has the *albino* gene (45). It has also been recently shown that alternative splicing of tyrosinase transcripts occurs in human as well as in murine melanocytes (37, 38, 56).

We have used these anti-peptide IgG to immunoprecipitate metabolically labeled proteins synthesized by B16 and JB/MS cells, two different but highly pigmented murine melanomas, and by melan-a and B10.BR normal murine melanocytes; all these lines were originally derived from C57Bl black mice (which are wild-type for the b and c loci). Virtually identical qualitative results were obtained in all experiments with these four cell lines and only the results with B10.BR cells are shown in Fig. 1. αPEP1 and αPEP7 IgG recognized metabolically labeled proteins under native conditions (arrow in Fig. 1A), but none of the other anti-peptide IgG recognized nondenatured protein(s). When identical metabolically labeled cellular proteins were denatured prior to immunoprecipitation, αPEP2 IgG now recognized a protein identical in electrophoretic properties to that recognized by αPEP1 IgG

TABLE I
Synthetic peptides used in the study

Peptide	Sequence	Derivation[a]
PEP1	CEDYEELPNPNHSMV-CO$_2$H	*brown*-Protein, COOH terminus
PEP2	QFPRECANIEALRR-CO$_2$H	*brown*-Protein, NH$_2$ terminus
PEP5	HFPRACASSKNLLA-CO$_2$H	*albino*-Protein, NH$_2$ terminus
PEP6	CCSSLWA-CO$_2$H	Truncated *albino*-protein, COOH terminus
PEP7	CDKDDYHSLLYQSHL-CO$_2$H	*albino*-Protein, COOH terminus

Antibody	Specificity of antisera[b]						Competitive binding[c]			
	None	PEP1	PEP2	PEP5	PEP6	PEP7	A	B	C	D
αPEP1	0.01	1.23	0.02	0.02	0.03	0.00	0.00	1.23	0.04	1.70
αPEP2	0.02	0.09	1.44	0.05	0.12	0.02	0.01	1.44	0.73	1.84
αPEP5	0.00	0.03	0.05	>2.00	0.03	0.01	0.00	>2.00	0.90	>2.00
αPEP6	0.00	0.00	0.02	0.01	0.95	0.00	0.01	0.95	0.17	0.82
αPEP7	0.00	0.00	0.00	0.00	0.01	1.35	0.00	1.35	0.06	1.32

[a] Peptide sequences for the b-protein, derived from pMT4, were taken from Ref. 31; for the c-protein, derived from MTY811, were taken from Ref. 35, and for the aberrantly terminated form of the c-protein, derived from Tyrs-33, were taken from Ref. 33.

[b] 0.1 μg of peptide as noted were bound to microtiter plates and antisera as indicated (diluted 1:1000 in PBS) were quantified by ELISA as detailed under "Experimental Procedures."

[c] 0.1 μg of the relevant immunizing peptide were bound to microtiter plates; sera as noted (diluted 1:1000 in PBS) were assayed by ELISA following preincubation for 60 min at 23 °C, as follows: A, preimmune serum; B–D, immune sera with: no additions (B), 10 μg/ml of the immunizing peptide (C), or 10 μg/ml of an irrelevant peptide (D).

A) Nondenaturing conditions   B) Denaturing conditions

A) Isoelectric focusing   B) Proteolytic degradation
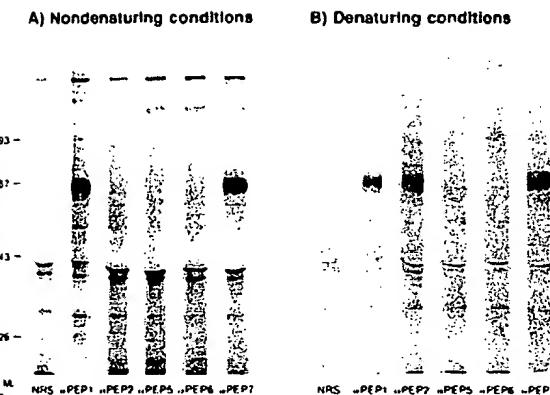
FIG. 1. **Immunoreactivity of peptide antibodies.** Normal murine melanocytes were labeled for 30 min with [^{35}S]methionine, chased for 60 min, solubilized in 1% Nonidet P-40/PBS and then immunoprecipitated, with or without prior denaturation, with the antibodies noted, as detailed under "Experimental Procedures." Specifically recognized proteins were separated by SDS-gel electrophoresis and visualized by autoradiography. M_r indicated are based on the migration of ^{14}C-labeled standard proteins. *NRS*, normal rabbit serum. *A*, nondenaturing conditions; *B*, denaturing conditions.

(arrow in Fig. 1*B*). As previously discussed (44), these results suggest that the amino terminus of the *b*-protein is buried in the native protein, and inaccessible to αPEP2, but that following denaturation, the epitope is exposed and immunoreactive. We were unable to detect any immunoreactivity of pulse chase labeled *c*-protein synthesized by any of these cell lines with αPEP5 or αPEP6 IgG (which should recognize the amino terminus) under any of the labeling and immunoprecipitation conditions employed in these studies; this probably results from both the low level of synthesis of the *c*-protein by these cells and also from the inaccessibility of the epitopes involved. The apparent contradiction that the *c*-protein was recognized by antibodies directed against its COOH terminus but not by those directed against its putative amino terminus might further indicate that cleavage of the signal peptide at the amino terminus occurs further into the molecule than proposed.

The M_r of the protein recognized by αPEP1 and αPEP2 IgG (a disperse band of ~66,000–71,000) agreed reasonably well with the predicted M_r 57,900 of the *b*-protein encoded by pMT4 (31), if one takes into account the heavily glycosylated nature of mammalian tyrosinase (57). The similar size of the protein recognized by αPEP7 agreed with the predicted M_r 58,600 of the *c*-protein encoded by MTY811 (35). Note that the electrophoretic mobility and disperse appearance of the *brown* and *albino* locus encoded proteins were virtually identical in these wild-type cells, and that they cannot be distinguished electrophoretically on the basis of their size.

When the bands immunoprecipitated by αPEP1 and αPEP7 IgG were resolved into two species, a sharp band at pH 6.5 and a disperse band at pH 5.8 *(arrows* in Fig. 2*A*); thus the *c*- and *b*-proteins were also indistinguishable on the basis of their intrinsic charge. The predicted pI values of the native proteins (*c*-protein, 5.98 and *b*-protein, 5.96) are reasonably close to the lower of those observed values, thus that band probably represents the *de novo* form of the proteins, which are heterogeneous by virtue of glycosylation. The protease sensitivies of the *b*- and *c*-proteins were significantly different, however, as detected by partial proteolysis following treatment with *S. aureus* protease (Fig. 2*B*). The *b*-protein was more readily degraded than the *c*-protein (especially noticeable at 1 μg/ml)
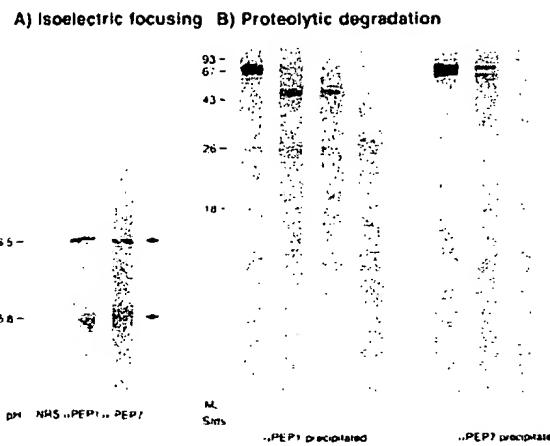
FIG. 2. **Characteristics of antigens recognized.** Normal murine melanocytes were labeled with [^{35}S]methionine, solubilized, and then immunoprecipitated with the antibodies noted, as detailed in Fig. 1. Specifically precipitated bands were cut out of the gels and analyzed by isoelectric focusing or by partial proteolytic degradation with 0, 1, 10, or 100 μg/ml *S. aureus* protease, as detailed under "Experimental Procedures." pH values indicated are from direct measurements on the focusing gels. *NRS*, normal rabbit serum. *A*, isoelectric focusing; *B*, partial proteolytic degradation.

A) DBA (brown)   B) MNT1 (human)
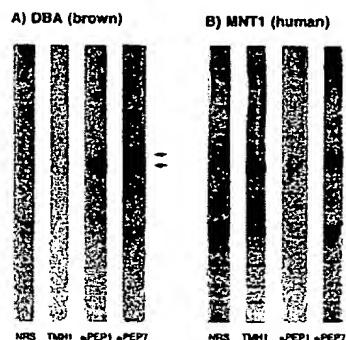
FIG. 3. **Cross-reactivity of peptide antibodies.** Cells as noted below were labeled and immunoprecipitated by antibodies as noted for Fig. 1, using nondenaturing conditions. *NRS*, normal rabbit serum. *A*, DBA (brown) cells; *B*, MNT1 (human melanoma cells).

and the pattern of fragments generated was distinct, further showing the specificity of the antibodies.

This noncross-reactive pattern of specificity can be further seen in the reactivities of these anti-peptide IgG with metabolically labeled proteins synthesized by DBA melanocytes. DBA cells are mutant at the *brown* locus and this mutation can be seen in the altered electrophoretic mobility of the *b*-protein produced by these cells (M_r is decreased by ~5000; *arrows* in Fig. 3*A*). Similar results were obtained with melanb cells, which are also mutant at the *brown* locus (not shown). It is interesting to note that both these independent mutations at the *brown* locus resulted in the loss of the epitope recognized by TMH1; it has been recently reported (58) that the brown mutation results from only 2 amino acid residue substitutions. In view of the difference in M_r noted in this study and previously (10), it seems likely that those substitutions result in aberrant glycosylation of the protein and the loss of the TMH1 epitope Note further here the distinct patterns of reactivity in the proteins recognized by the αPEP1 and αPEP7 IgG.

αPEP7 IgG recognized metabolically labeled proteins produced by normal human melanocytes or by MNT1 human

melanoma cells (*arrow* in Fig. 3*B*); this is reasonable since the peptide sequences of the *c*-proteins produced by murine and human melanocytes have been highly conserved (20), there are only 2 residue substitutions in their COOH-terminal peptides. On the other hand, αPEP1 and αPEP2 IgG did not recognize the *b*-protein expressed by human melanocytes. A *b*-protein analog is synthesized by human melanocytes since the *brown* locus specific monoclonal antibody TMH-1 cross-reacted with the human *b*-protein (59 and Fig. 2*C*). It has been recently reported (43) that the human melanoma antigen termed gp75 is the human analog of the murine *b*-protein, and this gene has now been cloned and sequenced (42, 43). Although the identity in the primary structure of the murine and human *b*-protein is greater than 90% overall, the carboxyl terminus of the human *b*-protein is truncated 11 residues before the murine carboxyl terminus, thus explaining the loss of reactivity of αPEP1 IgG with human melanocyte *b*-protein.

Expression of Proteins Analyzed by Western Blotting—We have used Western blotting methods to examine whether the *albino* or *brown* locus products might be preferentially expressed by normal or transformed melanocytes, since probes mapping to the *albino* locus were initially generated from normal melanocyte lines (32, 33, 35) while genes mapping to the *brown* locus were derived from transformed melanocyte lines (31, 34). We thus prepared extracts from cutaneous and ocular melanocytes from skin and eyes of newborn pigmented mice (as examples of normal melanocytes growing *in vivo*) and from B16 melanomas growing subcutaneously in mice (as examples of transformed melanocytes growing *in vivo*) for analysis by Western blot methodology. The results demonstrated many of the specificities noted above using metabolic labeling and immunoprecipitation protocols, but with several additional findings. Fig. 4*A* shows the distribution of tyrosinase isozymes in extracts of B16 cells and of normal ocular tissue from C57Bl mice that have been separated under nondenaturing conditions; DOPA oxidase activity is visualized by production of melanin following incubation with DOPA substrate. The major tyrosinase band had a M_r of ~70,000 and there was a minor band of ~150,000 (*arrows*); proteins in these bands were able to use tyrosine as a substrate as well as DOPA since incubation with [^{14}C]tyrosine also produced melanin, in this case visualized by autoradiography of the acid-insoluble product (53, 54). The specific activity of tyrosinase

in the B16 extract was significantly higher than in the eye extract (~5 times). As was noted in the metabolic labeling study shown above, αPEP1 and αPEP7 IgG specifically identified bands (*arrows* in Fig. 4*B*) of similar molecular mass to the 65-kDa tyrosinase band under these nondenaturing conditions (αPEP1 IgG also recognized the higher M_r tyrosinase band). Following denaturation of the proteins, αPEP5 (although not αPEP6) IgG also recognized proteins produced by normal melanocytes (*arrows* in Fig. 4*C*); this reactivity has never been detectable in extracts of melanocytes grown *in vitro*. A low M_r protein (~49,000) presumably encoded by pmcTyr3 (predicted M_r ~ 52,000) was recognized both in the normal and in the transformed cell extracts, while the full length protein encoded by MTY811 was only recognized in the normal C57Bl extract.

Catalytic Function of the Molecules—In a series of experiments, we have used immune affinity chromatography to isolate the proteins encoded by the *albino* and *brown* loci from B16 melanoma cells and have quantitated their melanogenic activities (Table II). In these experiments, we typically observed recovery of melanogenic activities from extracts of the B16 cells in a 1:2 ratio, which is consistent with earlier observations of the *b*-protein having about 30% of the total tyrosinase activity. In our initial experiments we attempted to elute activity bound to control and anti-peptide IgG complexed to protein A-Sepharose columns using excess peptide (*cf.* Protocol 1) although this approach did not work. However, elution with the acidic pH buffer supplied with the protein A-Sepharose kits did work; 15% of the tyrosine hydroxylase activity could be recovered from a single passage over the αPEP1 IgG column, which was similar to the 16% recovery reported earlier (44) using a different procedure. Similarly, recovery of DOPA oxidase and melanin formation activity showed 20–12% recoveries, respectively; the binding and recovery was specific since less than 1% of the activity was bound to control rabbit IgG complexed to protein A-Sepharose columns in similar fashion. Typically, tyrosinase would be irreversibly inactivated under the acidic conditions employed to elute these columns (pH 3.0); however, limited exposure at 4 °C to the deoxygenated buffer supplied with the protein A-Sepharose kits had little or no effect on enzyme activity if immediately dialyzed to restore physiological pH. In other experiments, we purified IgG recognizing PEP1 or
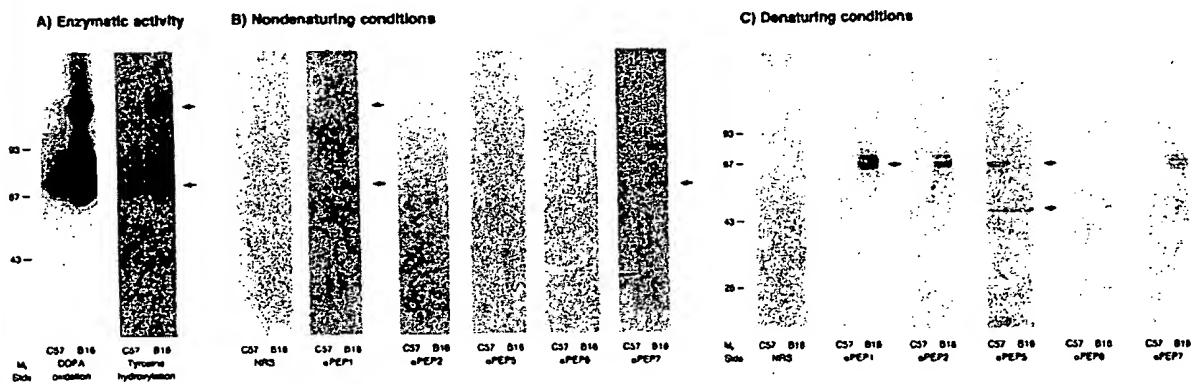

FIG. 4. **Reactivity of cellular proteins by Western immunoblotting.** Nonidet P-40/PBS extracts were made from B16 melanoma cells and from C57Bl newborn mouse retina/choroid; 50 μg of protein were electrophoresed in each lane and transferred to nitrocellulose sheets, as detailed under "Experimental Procedures." Enzymatic reactivity was visualized by incubation with DOPA substrate for melanin formation or with [^{14}C]tyrosine for production of radioactive melanin, and immune reactivities with anti-peptide IgG as detailed under "Experimental Procedures." *a*, enzymatic activity; *B*, nondenaturing conditions; *C*, denaturing conditions. *NRS*, normal rabbit serum.

TABLE II

Immune-affinity purification of melanogenic activities

Melanogenic activities, purified from crude Nonidet P-40 extracts of B16 melanoma cells by a single passage over 1-ml immune-affinity columns as noted, were determined as described under "Experimental Procedures." Assays were performed in triplicate and results presented are from a 1-h incubation at 37 °C; variations within experiments were always less than 10% and standard errors are not reported for the sake of simplifying the data. Percent activities found in each fraction are shown in parentheses. The unbound fraction indicates activity in the extract that was not retained by the column. NT, not tested.

Type of affinity column	Tyrosine hydroxylase	DOPA oxidase	DOPAchrome isomerase	Melanin formation
	dpm/h	$\Delta A_{475}/h$	ΔA_{475}	*dpm/h*
Protocol 1 α-Peptide IgG linked to protein A-Sepharose columns				
Control IgG				
Unbound	49,610 (99%)	0.17 (100%)	NT	58,048 (99%)
PEP1 eluted	0 (0%)	0.00 (0%)	NT	0 (0%)
Acid pH eluted	251 (<1%)	0.00 (0%)	NT	563 (<1%)
αPEP1 IgG				
Unbound	43,440 (84%)	0.12 (80%)	NT	34,286 (87%)
PEP1 eluted	296 (<1%)	0.00 (0%)	NT	86 (<1%)
Acid pH eluted	7,730 (15%)	0.03 (20%)	NT	4,706 (12%)
Protocol 2 α-Peptide IgG covalently linked to Sepharose columns				
αPEP1 IgG				
Unbound	43,317 (83%)	0.40 (96%)	−0.12 (100%)	40,054 (98%)
1% SDS eluted	5,090 (10%)	0.01 (2%)	0.00 (0%)	864 (2%)
Acid pH eluted	3,726 (7%)	0.01 (2%)	0.00 (0%)	0 (0%)
αPEP7 IgG				
Unbound	32,939 (68%)	0.24 (73%)	−0.05 (100%)	24,400 (57%)
1% SDS eluted	15,100 (31%)	0.09 (27%)	0.01 (0%)	18,227 (43%)
Acid pH eluted	68 (<1%)	0.00 (0%)	0.01 (0%)	0 (0%)

PEP7 and bound them covalently to affinity columns (cf. Protocol II) with similar results. In the latter protocols we eluted the columns with 1% SDS buffer prior to washing with the acidic elution buffer; in both cases, most of the bound activity was eluted with the SDS buffer. We have since found that SDS at concentrations above 0.1% is sufficient to dissociate these anti-peptide IgG from their bound ligands, while levels of 0.01% or less (as used in the protocols for radioimmunoprecipitation analysis) do not. Both the b-protein and the c-protein immune affinity purified in this manner contained tyrosine hydroxylase, DOPA oxidase and melanin formation activities in similar proportions, and neither could utilize DOPAchrome as a substrate (Table II).

In view of the inability of some earlier studies to demonstrate significant tyrosinase activity for the b-protein, it was essential that the possibility of cross-contamination between these proteins be eliminated. We have employed three protocols in addition to the experiments previously discussed to elaborate this point. First, we have used immunodepletion protocols to demonstrate that αPEP1 and αPEP7 IgG recognize distinct, immunologically non-crossreactive and, at least in the solubilized state, nonassociating proteins (Fig. 5A). Following three rounds of absorption with normal rabbit sera, both immunoreactive proteins remained in the supernatant (labeling of the c-protein was relatively low in these experiments, cf. "Discussion"). Sequential absorption with αPEP1 or αPEP7 IgG however, removed only the band expected (~95% of immunoreactive components were removed in the first absorptions, 5% in the second, and none in the third). Second, proteins immunoprecipitated by the peptide antibodies were recovered and subjected to another round of antibody complexing and immunoprecipitation (Fig. 5B). The results demonstrated that labeled protein initially recognized by αPEP1 IgG could be subsequently recognized only by αPEP1 IgG, and conversely, protein immunoprecipitated by αPEP7 could only be reimmunoprecipitated by αPEP7 IgG. In these experiments, the yield was between 50 and 70% in the positive

reactions and 0% in the negative reactions. Third, we have analyzed the original extracts applied to, and the fractions eluted from, the immune-affinity columns, using Western blot methods (Fig. 5C). There is some leakage of the IgG from these covalently bound columns, which leads to a high background in the column fractions tested by normal serum, αPEP1 and αPEP7 IgG (these bands are not observed in the original homogenate, as expected). It can be seen that the sample which eluted from the αPEP1 column contained material only immunoreactive with αPEP1 IgG, while material eluted from the αPEP7 column could be recognized only by the αPEP7 IgG.

Repetitions of these studies (summarized in Fig. 6) have yielded average recoveries from extracts of B16 cells of tyrosine hydroxylase activities of 11 and 28%, DOPA oxidase activities of 58 and 92%, and of melanin production activities of 7 and 24%, by αPEP1 and αPEP7 IgG, respectively. In related studies we have found that there was no demonstrable synergism of activity between these immunopurified enzyme preparations; when premixed prior to assay, their enzymatic potentials were simply additive. The only difference between these proteins noted to date is the relative lack of a lag period in catalytic activities of the c-protein, and a significant lag in the time course for melanogenic function which occurs with the b-protein (visible in the DOPA oxidase kinetics shown in Fig. 7 and in both the [³H]tyrosine and the [¹⁴C]tyrosine assays, not shown).

DISCUSSION

The extensive amino acid sequence identity in the b-protein and the c-protein expressed by murine and human melanocytes is striking (cf. Fig. 4 in Ref. 20 and Fig. 6 in Ref. 44); each of these genes has ~90% sequence identity between murine and human species. Both genes are closely related: more than 45% of the residues in each sequence have been conserved throughout exons 1–4, which contain the catalytic domains. However, the b-protein and c-protein differ signifi-

A) Immunodepletion protocols

## B) Re-Immunoprecipitation protocols

C) Western blot analysis
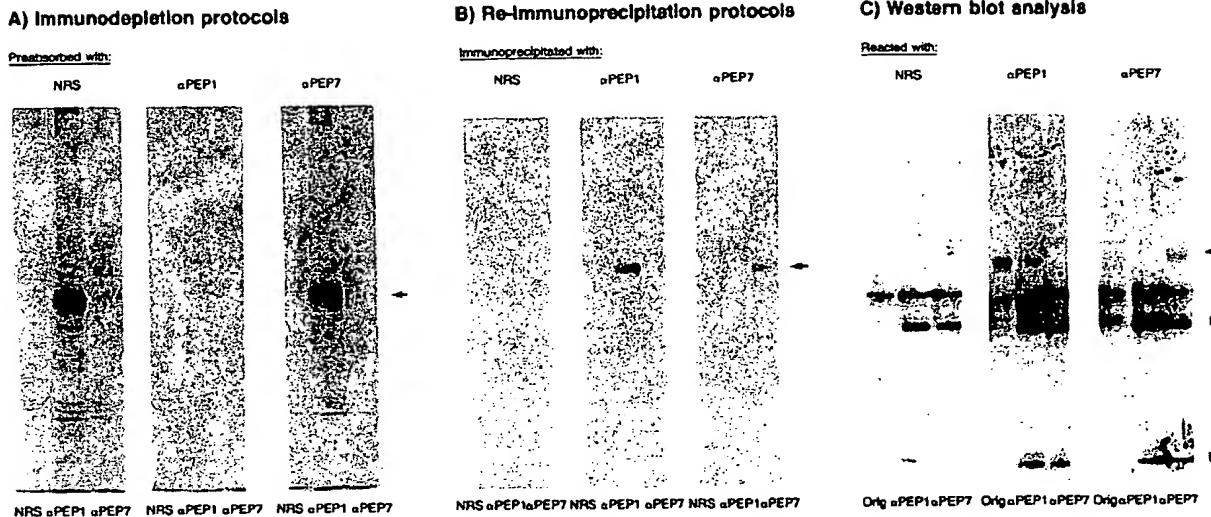
FIG. 5. Specificity of immune-affinity purification of melanogenic proteins. Melanocytes were radio-labeled and immunoprecipitated as detailed for Fig. 1. *A*, immunodepletion protocols: extracts were sequentially preabsorbed three times with IgG (as listed at the *top*), and then immunoprecipitated with antibodies (as noted at the *bottom*). *B*, reimmunoprecipitation protocols: immunoprecipitates recovered from antisera listed as detailed for Fig. 2 were subjected to immunoprecipitation with the antibodies noted. *C*, Western blot analysis: aliquots from the original extract applied to the immune-affinity columns (*Orig*), or specifically eluted from the αPEP1 or αPEP7 columns (as noted at the *bottom*) were electrophoresed, transferred to nitrocellulose filters, and examined for antibody reactivity, as detailed under "Experimental Procedures." Positions of the heavy (*H*,) and light (*Lt*) IgG chains, which slowly elute from the affinity columns, and are detected by the secondary antibodies (as listed at the *top*). *NRS*, normal rabbit serum.
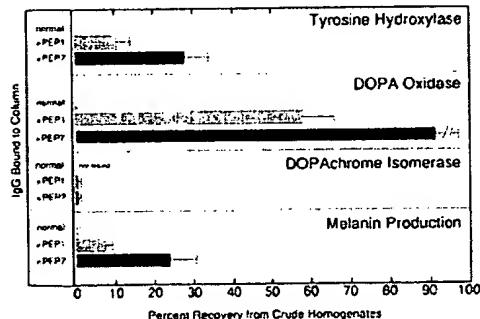
FIG. 6. Summary of immune-affinity purification of melanogenic activities. Results on purification of melanogenic activities from repeated immune-affinity purifications in different experiments are shown as the mean percent recoveries from crude extracts of B16 melanoma cells ± S.E. (where applicable). Experiments were performed as detailed for Table II. *n* = 1 for control IgG; *n* = 7 for αPEP1 IgG; and *n* = 5 for αPEP7 IgG.

FIG. 7. Time course of DOPA oxidase assays on immune-purified tyrosinase. Proteins isolated by immune-affinity purification by αPEP1 and αPEP7 IgG (as detailed for Table II) were used for determination of DOPA oxidase activity over a 6-h period, as detailed under "Experimental Procedures."

cantly at their carboxyl termini (exon 5), which contain the transmembrane-spanning and cytoplasmic orientation determinants. These highly conserved molecules have many of the structural features expected of tyrosinase, such as two copper binding sites required for catalytic activity (and where the sequence identity approaches 70%), potential glycosylation sites, and orientation of the catalytic site away from the cytoplasm, that is, within the melanosome, a membrane-bound cytoplasmic organelle. All 15 of the cysteine residues have been conserved among these proteins, 10 of the 11 tryptophans have also been conserved, and the three histidines potentially responsible for binding a copper atom in each of the two copper binding sites have also been conserved, along with most of the other hydrophobic residues in those regions. The extent of homology between the *brown* and *albino*

genes and the recently cloned TRP2 gene,[2] which appears to represent yet another member of the tyrosinase family, remains to be determined, although it is known that the COOH terminus of TRP2 is completely different from the COOH termini of the *b*- and *c*-proteins,[2] and thus should not cross-react with any of the antibody probes used in this study.

The actual capacity of these molecules to function catalytically as tyrosinase, *i.e.* to contain tyrosine hydroxylase and DOPA oxidase activities, has previously been demonstrated for the *b*-protein in murine melanoma cells *in vivo* and *in vitro* (46, 60). Interestingly, both those studies reported the removal of only about 30% of total tyrosinase activity from extracts using tyrosinase-specific antibodies; whether the tyrosinase remaining (~70%) was not recognized due to the deletion of relevant epitopes by mechanical means (*e.g.* by endogenous proteases) or whether it was due to the presence of other distinct tyrosinases could not be determined at that time. However, the abilities of the *b*-protein and the *c*-protein

to function catalytically have recently been demonstrated by immunological and transfection techniques, respectively (36, 38, 41, 44). Tyrosinase is an unusual enzyme in that it contains two, and possibly a third, distinct catalytic activities. Thus it seemed reasonable to speculate that perhaps each of those enzymatic activities actually resided on separate molecules (such as the *b*- and *c*-proteins) which by nature of their structural similarity were previously inseparable and indistinguishable by most, if not all, biochemical purification techniques. In fact, we can only discriminate between these two proteins using immunologic reagents. In this study, we have used immune-affinity chromatography with peptide-specific antibodies to purify both proteins and have been able to establish their catalytic potentials from the same cell type in order to more accurately measure their contribution to observed melanogenesis, their substrate specificity and any potential interactions. Both proteins were found to be competent for both of the catalytic activities ascribed to tyrosinase. However, although the *b*-protein accounts for approximately 20–30% of the total tyrosinase activity present in these melanocyte populations, the *b*-protein is typically synthesized, and is present, in a higher concentration than the *c*-protein (10, 43, and our results). This shows that the specific catalytic activity of the *c*-protein must be significantly higher than the *b*-protein for these melanogenic activities. It may be that the lower specific catalytic activity of the *b*-protein is one reason that previous attempts to demonstrate tyrosinase activity by the *b*-protein using transfection protocols were unsuccessful, since the catalytic function of even the *c*-protein expressed by those transfected cells was relatively low (38, 45). These results also help to explain the previous contentions (as outlined in the Introduction) over the assignment of the *albino* or the *brown* locus as the structural locus for tyrosinase since both loci encode proteins with catalytic potential; the fact that the *b*-protein has at least low levels of tyrosinase activity is consistent with previous studies showing significant but low levels of tyrosinase activity in albino animals, even though no melanin was formed (27–30).

The fact that *c*-protein reactivity with αPEP5 can be easily detected in *in vivo* extracts of cells by Western blotting, but not by short (<6 h) pulse metabolic labeling suggests a slow rate of synthesis yet a highly stable nature for this protein. Such results are consistent with earlier studies showing an *in vivo* half-life for tyrosinase of greater than 12 h (1, 46, 61). Halaban and coworkers (25) have shown that rates of synthesis and degradation of tyrosinase can vary dramatically in different pigment mutants, and have a profound effect on pigment production by those tissues. Throughout the course of our studies, we have been unable to identify any reactivity with αPEP6 IgG, which should specifically recognize the Tyrs-33 transcript. This transcript contains a large deletion through one of the putative copper binding sites at exon 3 (45), and also apparently has a deletion at the carboxyl terminus which results in a frameshift mutation and a unique carboxyl terminus. Our results suggest that translation of this misspliced mRNA either does not occur (or occurs at extremely low frequency) in these cells, and/or that the protein produced has a very short half-life. Other products which occur as the result of misspliced mRNA from the *albino* locus (45), which maintain the correct carboxyl terminus, were detectable in this study.

In related experiments, we have also used metabolic labeling and Western blot methods to examine the immunoreactivity of melanocyte proteins with these antibodies, before and after treatment with MSH (not shown). Similar results were found to the results outlined above, *i.e.* that αPEP1 and αPEP7 IgG

recognized proteins expressed by MSH-treated cells under native conditions, and that αPEP2 IgG recognized the same protein only after the proteins had been denatured. Although MSH treatment resulted in a more than 30-fold increase in tyrosinase activity by these cells (46), the rates of *b*- and *c*-proteins synthesized were not significantly increased (<2-fold); these results are consistent with a recent study which used Northern blot analyses to show that increases in tyrosinase activities in response to MSH stimulation are not regulated at the level of transcription (62). These results are consistent with previous studies on the activation of latent tyrosinase being the primary mechanism whereby MSH stimulates melanogenesis (1, 46, 61, 63). Throughout our experiments, the expression of the *b*-protein has been relatively stable, while rates of *c*-protein synthesis have been variable, ranging from levels comparable to the *b*-protein (as seen in Fig. 1) to levels 10–20 times lower (as seen in Fig. 5); future studies will be directed as characterizing factors which influence the relative balance of expression of these two proteins, as well as their interactions.

There are several critical implications of this work which demand a rethinking of our concepts of mammalian melanogenesis. First, we must now acknowledge the fact that melanin production does not depend on a single enzyme moiety. In addition to the homologous genes examined in this report, several other, immunologically cross-reactive gene products have recently been reported (34, 37, 64) including TRP2,[2] and although detailed information is not yet available to predict the extent of those sequence identities, there can be no doubt that a family of pigment-related, tyrosinase homologous genes is emerging. It has been clearly demonstrated that expression of tyrosinase mRNA alone does not ensure pigment production since normal amounts of tyrosinase message have been detected in amelanotic cells (36, 38). In addition to tyrosinase(s), several additional factors have been recently reported which function to inhibit or stimulate melanin production; these operate distal to tyrosinase activity and may be other critical regulatory controls (4–16). Second, the interrelationships of the *albino* and *brown* loci in the control of mammalian pigmentation have in some ways become more muddled rather than clarified due to the elucidation of the proteins encoded by those loci. There is no doubt that the *albino* locus controls whether pigment is produced by melanocytes and that the *brown* locus specifies the nature of the melanin produced (black *versus* brown). Conventional wisdom has suggested that either locus might encode a melanogenic regulator of some kind, or an enzymatically functional molecule, but not that both loci might encode such highly conserved catalytically functional molecules. Complementation between the products of the *albino* and *brown* loci has been recently shown to produce normal black melanin (65). One obvious possible explanation, *i.e.* that one molecule has tyrosine hydroxylase activity while the other has DOPA oxidase activity, does not seem to be the case since our concerted efforts to demonstrate this have failed; each of the two proteins seems to be competent for both catalytic functions. Studies are now underway in our and in other laboratories to more definitively examine the proteins and catalytic activities expressed by melanocytes derived from different pigment mutations; such studies should eventually define the mechanisms involved in the interaction of these pigment related genes. It may be that we have not yet defined the optimal substrates and conditions to assess the interactions of these two closely related enzymes. It has been recently proposed that metal ions and catalase and/or peroxidase activities might also be involved in the regulation of melanin production (9, 10, 13, 14); Halaban and Moellmann

(10) have reported that the *b*- and *c*-proteins have two potential iron-binding sites which, combined with the fact that iron has recently been shown to be critical to stimulation of tyrosinase activity (14), suggests that the iron-binding potential may be as important to the catalytic function of tyrosinase as its copper-binding potential. Recent work demonstrating that the albino mutation results from the mutation of one of these iron binding sites (Cys[90] to Ser) (66) and that the *brown* mutation similarly results from a mutation in the same iron binding site (Cys[87] to Tyr) (58) further underscores the critical nature of this metal-binding site. Third, recent studies have demonstrated that mRNAs transcribed from these loci are aberrantly processed, that is, they are misspliced, to varying degrees in murine (40, 45) and in human (37, 38, 56) melanocytes. The inability of several of these misprocessed proteins to function catalytically has been shown (36, 38, 41) but whether this splicing step might also function in the regulation of melanogenesis, either directly by controlling the amount of functional enzyme, or indirectly if the misspliced proteins turn out to be functional themselves (*e.g.* as competitive inhibitors), is another intriguing possibility. Our antipeptide IgG, which recognize the aberrant products, will be one approach to answering this question. Fourth, and perhaps most importantly, is the alteration of gene expression and the increased misprocessing of mRNA transcripts by transformed melanocytes. It has been known for some time that neoplastic cells have altered patterns of protein expression; many of these altered proteins can function as tumor-specific markers, and in some cases, as tumor rejection antigens (67). Although many laboratories have been involved in the effort to identify and characterize tumor specific antigens, and the genetic mechanisms responsible for their production, none to date have been more than partially successful. The alteration in the patterns of expression of the *brown* and the *albino* loci by normal and transformed melanocytes may well turn out to be an optimal system for such studies, since the genes involved have already been cloned, and mapped, and the products of those genes have defined (albeit confusing at this time) functions in mammalian cells.

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Eumelanin Biosynthesis Is Regulated by Coordinate Expression of Tyrosinase and Tyrosinase-Related Protein-1 Genes

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Melanin is specifically produced in melanocytes. The pathway for melanin biosynthesis is regulated by a number of melanocyte-specific proteins, including tyrosinase and tyrosinase-related protein-1 (TRP-1, *b* locus protein). To understand the regulation of melanogenesis, we examined tyrosinase activities, mRNA levels of tyrosinase and TRP-1, and eumelanin and pheomelanin contents in mouse B16-F1 melanoma cells after they had been treated with some melanotropic reagents. Cholera toxin, α-melanocyte-stimulating hormone, and dibutyryl cyclic AMP increased tyrosinase activity and stimulated eumelanin biosynthesis. These reagents elevated intracellular cAMP levels. In contrast, 12-*O*-tetradecanoylphorbol 13-acetate reduced tyrosinase activity and eumelanin synthesis. In all cases, the mRNA levels of tyrosinase and TRP-1 changed in parallel with tyrosinase activity and eumelanin content. TRP-1 was induced simultaneously with tyrosinase, although its inducibility was lower than that of tyrosinase. These results suggest that the expressions of tyrosinase and TRP-1 genes are coordinately regulated by melanotropic reagents through cAMP-dependent protein kinase and protein kinase C in mouse B16-F1 cells, and that their coordinate expression causes eumelanin biosynthesis. © 1993 Academic Press, Inc.

INTRODUCTION

Melanin determines the skin and hair color of animals and is especially important for protecting skin from harmful ultraviolet irradiation in humans. It is synthesized specifically in melanocytes of hair follicles, eyes, and the basal layer of epidermis. From the chemical structure, there are two classes of melanin pigments, eumelanin and pheomelanin. Eumelanin is a dark-brown to black pigment which is insoluble in acid and alkali and contains no sulfur (0–1%). Pheomelanin is a yellow to reddish-brown pigment which is soluble in al-

kali and contains sulfur (9–12%) [1]. Eumelanin is more abundant and is an essential pigment in mammals.

Melanin biosynthesis occurs through sequential steps known as the Mason–Raper pathway [2] and is regulated by many melanotropic stimulations. Many melanocyte-specific proteins are involved in melanogenesis. Recently several melanocyte-specific cDNA clones encoding tyrosinase and other proteins have been isolated and characterized [3]. Tyrosinase (EC 1.14.18.1) is a rate-limiting and most important enzyme in melanogenesis, which catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPA to DOPAquinone [2, 4]. The tyrosinase gene is mapped to the *c* locus on chromosome 7 in mice and to chromosome 11 in humans [5]. It has been found that the albino, himalayan, and chinchilla mutants are caused by point mutations of tyrosinase [6–11].

The two novel cDNAs, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2), were also isolated and identified as tyrosinase-associated proteins [12, 13]. They have ~40% amino acid homology with tyrosinase and have the same structural features, including two copper binding sites, two cysteine-rich regions, a signal peptide, and a transmembrane domain [13]. The TRP-1 gene is mapped to the *b* locus on mouse chromosome 4, and the point mutation of TRP-1 causes the brown mutant [14, 15]. The eumelanin content in the skin of brown mutant mice was much less than that of wild-type mice, but the pheomelanin content was the same [16]. Thus, it is suggested that the function of TRP-1 is closely related to eumelanin biosynthesis. TRP-2 has been demonstrated to have DOPAchrome tautomerase activity, which converts DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [17]. This enzyme controls the proportion of carboxylated subunit in eumelanin. The gene is mapped to the *slaty* locus on mouse chromosome 14 [13]. The mutant shows dark-gray/brown skin colors. It is probable that, in addition to tyrosinase, these novel melanocyte-specific proteins play important roles for melanogenesis. Their significance in the regulation of melanogenesis has only begun to be elucidated; however, much remains to be clarified.

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33

In this paper we report that in mouse B16-F1 melanoma cells expressions of tyrosinase and TRP-1 genes are induced coordinately by melanogenic reagents and repressed also coordinately by 12-O-tetradecanoylphorbol-13-acetate (TPA). The possibilities that gene expressions of tyrosinase and TRP-1 are controlled by a common regulatory mechanism through cAMP-dependent protein kinase and protein kinase C and that the two proteins have synergistic roles in melanogenesis are discussed.

METHODS

Chemicals. Cholera toxin, α-melanocyte-stimulating hormone (α-MSH), dibutyryl cyclic AMP (dbcAMP), and TPA were purchased from Sigma Co. (St. Louis, MO). L-[3,5-³H]Tyrosine, [α-³²P]dCTP, and the cyclic AMP assay system were from Amersham Japan Ltd. (Tokyo, Japan). The Bio-Rad protein assay system was from Japan Bio-Rad Laboratories Ltd. (Tokyo, Japan). The random primer DNA labeling kit was from Takara Shuzo Co. (Kyoto, Japan). Celite 545 was from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Cell culture conditions. Mouse B16-F1 melanoma cells were obtained from American Type Culture Collection. Every 1 or 2 months new cultures were started from frozen stock to prevent phenotypic changes of the cells. Cells were cultured in DME/F12 culture medium supplemented with 200 units/ml penicillin G, 0.1 mg/ml streptomycin sulfate, and 10% fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO₂. Viable cell numbers were counted by the 0.15% trypan blue exclusion method with the use of a hemocytometer.

DOPA oxidase activity. This assay was done according to the method described by Jara *et al.* [4] with some modifications. Subconfluent cells cultured in 90-mm culture dishes were lysed with 4 ml of 0.5% deoxycholic acid, 50 mM Tris-Cl (pH 7.5). These cell lysates were used as samples for the assays of DOPA oxidase and tyrosine hydroxylase activities and melanin contents. Each 0.6-ml sample was incubated with 1.8 ml of 0.17% Triton X-100, 0.1 M KH₂PO₄ buffer (pH 6.8), and 0.6 ml of 5 mM L-DOPA at 37°C. OD₄₇₅ ₙₘ due to DO-PAchrome formation was monitored by a spectrophotometer (Gilford 250).

Tyrosine hydroxylase activity. This assay was also done according to the method described by Jara *et al.* [4]. Each 10 μl of sample was incubated at 37°C for 1 h with 10 μl of 0.1 M KH₂PO₄ buffer (pH 6.8), 10 μl of antibiotic mixture (1 mg/ml chloramphenicol, 1 mg/ml cyclohexamide, 1000 units/ml penicillin G, 0.1 mg/ml bovine serum albumin, 0.05 mM L-DOPA, 1 μl of L-[3,5-³H]tyrosine (1 μCi/μl, 50 Ci/mmol), and 18 μl of distilled water. After incubation, 0.95 ml of 2.5% trichloroacetic acid containing activated charcoal (65 mg/ml) and Celite 545 (65 mg/ml) was added to the sample and agitated continuously for 1 h at room temperature. After centrifugation at 10,000 rpm for 10 min, 400 μl of supernatant was taken and counted for radioactivity using a liquid scintillation counter (Packard Tri-Carb 4530).

Melanin content. The eumelanin and pheomelanin contents in samples were analyzed by the method of Ito *et al.* [1]. The eumelanin and pheomelanin contents were calculated on the basis that 1 ng of pyrrole-2,3,5-tricarboxylic acid and 1 ng of aminohydroxyphenylalanine roughly correspond to 50 ng of eumelanin and 5 ng of pheomelanin, respectively.

Protein content. Protein contents in samples were measured using the Bio-Rad protein assay system.

Cyclic AMP assay. Intracellular cAMP levels were measured according to the protocol of Amersham's cAMP assay system by the method of radioimmunoassay. The cAMP was extracted from cells with ice-cold 65% ethanol.

Isolation of RNA. Total cellular RNA was isolated according to the rapid isolation method [18]. Subconfluent cells in 90-mm plates were washed twice with phosphate-buffered saline and then 4 ml of 10 mM EDTA (pH 8.0), 0.5% SDS was added. The lysate was collected by scraping into a 50-ml tube and combined with 4 ml of 0.1 M sodium acetate (pH 5.2), 10 mM EDTA (pH 8.0). The tube was shaken with 8 ml of phenol (equilibrated with water) and centrifuged at 3400 rpm for 40 min at 4°C. The water phase was transferred to a fresh tube, and total cellular RNA was precipitated twice with ethanol and the amount was measured with a spectrophotometer (Hitachi U-2000).

Northern blot analysis. Northern blot analysis was performed according to the method described [19]. Each 7 μg of total cellular RNA was electrophoresed through a 1% agarose gel containing 6% formaldehyde. The RNA was completely transferred to a nylon membrane filter. The filter was hybridized with ³²P-labeled probe in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% SDS, 20 μg/ml herring sperm DNA at 42°C for 16 h. The hybridized filter was washed with 0.1× SSC, 0.1% SDS at 50°C and then autoradiographed for 7-90 h at −80°C with an intensifying screen. The density of each band of the autoradiograph was measured with an enhanced laser densitometer (LKB Ultroscan XL).

Probes. Radiolabeled probe (sp act 1–2 × 10⁹ cpm/μg DNA) was made using the random primer DNA labeling kit with [α-³²P]dCTP. The DNA probes used are as follows: tyrosinase, SpAMT γ 1 (mouse tyrosinase gene exon 1) [6]; TRP-1, pMT4 (mouse TRP-1 cDNA) [12]; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pSPGAPDH (rat GAPDH cDNA) [20]; and ribosomal protein L35a, pRL35a (rat L35a cDNA) [21].

RESULTS

Effects of Melanotropic Reagents on Tyrosinase Activity

We examined the effects of cholera toxin, α-MSH, dbcAMP, and TPA on DOPA oxidase activity and cell proliferation of B16-F1 melanoma cells. DOPA oxidase activity, which catalyzes the conversion of DOPA to DOPAquinone, is characteristic of tyrosinase activities. As shown in Figs. 1A and 1B, cholera toxin and α-MSH increased DOPA oxidase activity and suppressed cell growth in a dose-dependent manner. Both cholera toxin and α-MSH were effective at the concentrations higher than 10⁻¹¹ and 10⁻⁸ M, respectively. DbcAMP increased DOPA oxidase activity at concentrations from 0.5 to 2 mM (Fig. 1C). In contrast, TPA reduced DOPA oxidase activity dose dependently, but did not affect cell proliferation (Fig. 1D). The effect of TPA on DOPA oxidase activity was observed at concentrations higher than 1.6 × 10⁻⁸ M. The effective concentrations of the reagents used in this experiment were similar to those described in other papers [22-26]. It was interesting to observe that TPA suppressed the elevated level of DOPA oxidase activity caused by cholera toxin treatment antagonistically (Fig. 1E). A similar effect of TPA was also observed against α-MSH and dbcAMP (data not shown). Therefore, it is suggested that TPA has an antagonistic effect against cholera toxin, α-MSH, and dbcAMP. The time courses of the effect of cholera toxin (10⁻⁹ M) in the presence or absence of TPA (1.6 × 10⁻⁷ M) on DOPA oxidase activity are shown in Fig. 1F. Chol-
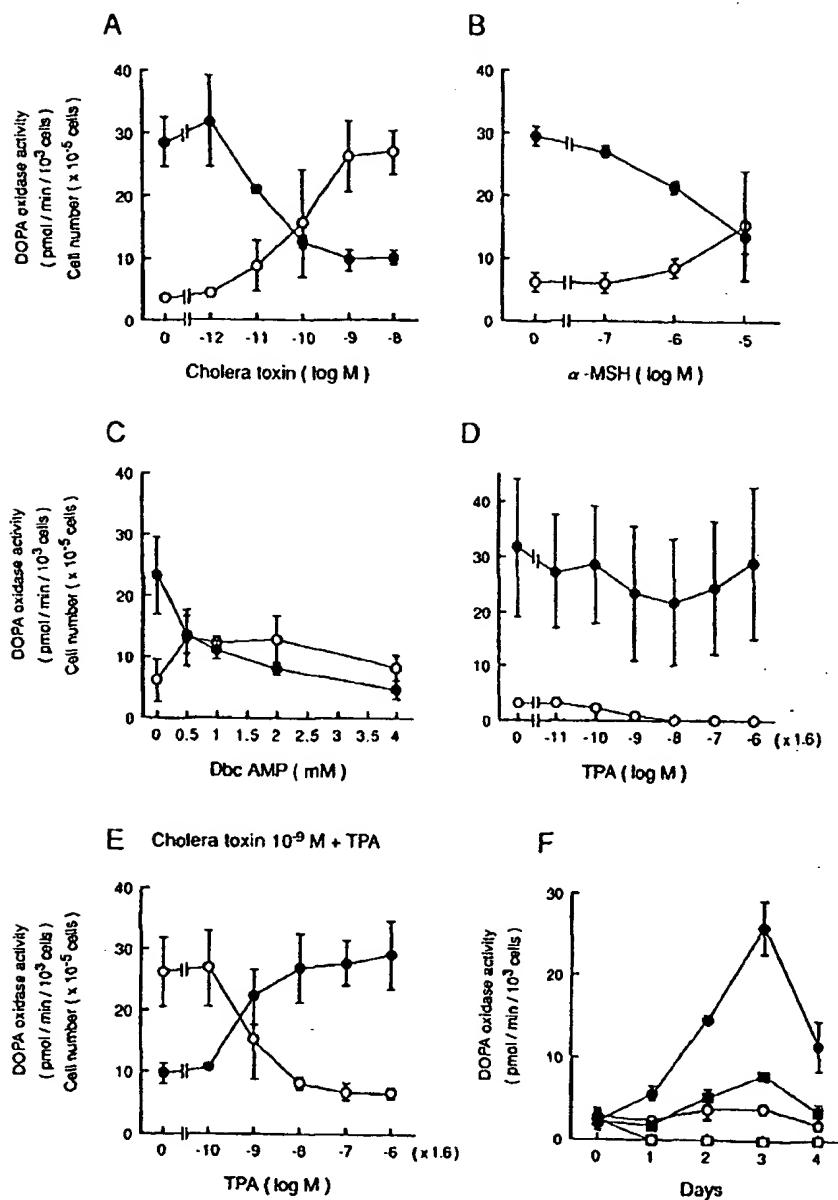
FIG. 1. Effects of cholera toxin, α-MSH, dbcAMP, and TPA on DOPA oxidase activity and on the proliferation rate of B16-F1 melanoma cells. (A-E) Cells (5×10^4) were seeded in 60-mm plates and the reagents were added to the culture medium. The cells were harvested after 3 days of culture, and viable cells were counted and DOPA oxidase activities were measured. The values represent means and the bars represent standard deviations ($n = 2$-6). O, DOPA oxidase activity; ●, cell number. (F) Culture conditions were the same as those of (A-E), except that the cells were harvested at Days 0-4. O, No treatment; ●, cholera toxin (10^{-9} M); □, TPA (1.6×10^{-7} M); ■, cholera toxin (10^{-9} M) + TPA (1.6×10^{-7} M).

era toxin increased DOPA oxidase activity, with a peak at the third day after treatment, and TPA reduced it to the great extent. The antagonistic effect of TPA against cholera toxin was observed throughout the entire period examined.

Effects of Melanotropic Reagents on Intracellular cAMP Levels

It is reported that cholera toxin and α-MSH activate adenylate cyclase, thus resulting in the elevation of in-

TABLE 1

Intracellular Cyclic AMP Levels in Cells Treated with Cholera Toxin, α-MSH, and TPA

Treatment	Cyclic AMP levels (fmol/10⁴ cells)					
	0 h	0.5 h	4 h	6 h	24 h	48 h
None	19.2 ± 3.03	nd	nd	23.2 ± 3.88	29.9 ± 2.07	22.9 ± 8.12
TPA	nd	22.7 ± 3.05	23.6 ± 0.71	nd	32.9 ± 4.03	18.6 ± 7.09
á-MSH	nd	3990 ± 1290	nd	62.0 ± 35.0	58.0 ± 10.6	36.0 ± 21.1
α-MSH + TPA	nd	6990 ± 1940	nd	36.0 ± 1.41	60.2 ± 5.37	16.1 ± 0.71
Cholera toxin	nd	nd	1450 ± 659	nd	667 ± 260	nd
Cholera toxin + TPA	nd	nd	1620 ± 236	nd	473 ± 251	nd

Note. Values are given as means ± standard deviations (n = 2–6). TPA, 1.6×10^{-7} M; α-MSH, 10^{-9} M; cholera toxin, 10^{-9} M. nd, not determined.

tracellular cAMP levels [22, 27]. It is also reported that TPA activates protein kinase C and can modify cAMP levels in some cell lines [28, 29]. In order to clarify whether melanogenic reagents used in this experiment have an activating effect on adenylate cyclase and whether TPA also has an effect on the enzyme, we measured intracellular levels of cAMP before and after treatment of the reagents in B16-F1 cells. Table 1 shows the results. α-MSH elevated the cAMP level 200-fold higher than that in control cells (at 0.5 h). The cAMP levels of cholera toxin-treated cells were also elevated 30- to 75-fold (at 4 and 24 h). Treatment with TPA alone showed no effect on cAMP levels. However, the cAMP level of the cells treated with α-MSH + TPA (at 0.5 h) was higher than that of cells treated with α-MSH alone ($P < 0.05$, t test). In this case, it appeared that α-MSH and TPA synergistically elevated the cAMP level in the treated cells. The reason for this phenomenon is unknown. Whether TPA emphasized the activating effect of α-MSH on adenylate cyclase or inhibited phosphodiesterase activity remains to be clarified. When TPA was added with cholera toxin, no additional effect on the elevation of cAMP was observed at each time examined. The facts indicate that TPA exerts an antagonistic effect against α-MSH and cholera toxin on DOPA oxidase activity without suppressing the cAMP levels. These results suggest that tyrosinase activity is induced by the elevation of intracellular cAMP, presumably through cAMP-dependent protein kinase, and reduced by TPA through protein kinase C without affecting the cAMP levels.

Effects of Melanotropic Reagents on Eumelanin and Pheomelanin Contents and the mRNA Levels of Tyrosinase and TRP-1

To determine the effects of these melanotropic reagents on the induction of tyrosinase and TRP-1 genes and also on melanin production, we examined the changes in mRNA levels of tyrosinase and TRP-1 and of eumelanin and pheomelanin contents at 48 h after treatment with reagents. Each reagent was used at its

effective concentration (10^{-9} M cholera toxin, 10^{-6} M α-MSH, 0.5 mM dbcAMP, and 1.6×10^{-7} M TPA). The specific activities of tyrosine hydroxylase and DOPA oxidase are shown in Fig. 2A. The changes in these two enzyme activities were parallel to each other. The ratios of the values between two assays were consistent with those previously reported [4]. TPA reduced the levels of both enzyme activities to 25–33% of those of nontreated cells. Cholera toxin, α-MSH, and dbcAMP increased the activities to 130–188%. In the presence of TPA, this stimulatory effect of melanogenic reagents on the enzyme activities was abolished. The eumelanin and pheomelanin contents in the cells after treatment with the melanotropic reagents are shown in Fig. 2B. Here again, the melanogenic reagents increased the eumelanin contents (190–500% of nontreatment levels) and TPA antagonistically reduced eumelanin production (46% of nontreatment levels), the change in which was almost parallel to that in tyrosinase activities. On the other hand, pheomelanin contents were not significantly changed. Eumelanin biosynthesis is actually regulated in response to melanotropic stimulations. Figure 3 shows the mRNA levels of tyrosinase and TRP-1 in the cells before and after treatment with melanotropic reagents, as determined by Northern blot analysis. GAPDH and ribosomal protein L35a were used as internal standards. It can be clearly seen that the mRNA levels of tyrosinase and TRP-1 change in parallel with tyrosinase activity and eumelanin contents (Figs. 3A and 3B). Cholera toxin, α-MSH, and dbcAMP elevated the tyrosinase mRNA level to 340–550% and the TRP-1 mRNA level to 200–300% of that of nontreated cells. The mRNA levels of GAPDH and L35a were limited maximally to a 170% increase. Treating the cells with TPA caused a reduction in the mRNA levels of tyrosinase and of TRP-1 to 26 and 41% of nontreatment levels, respectively. These results indicate that gene expressions of tyrosinase and TRP-1 are actually induced by melanogenic stimulations and that the inducibility of tyrosinase is higher than that of TRP-1. Figure 3C

A

B
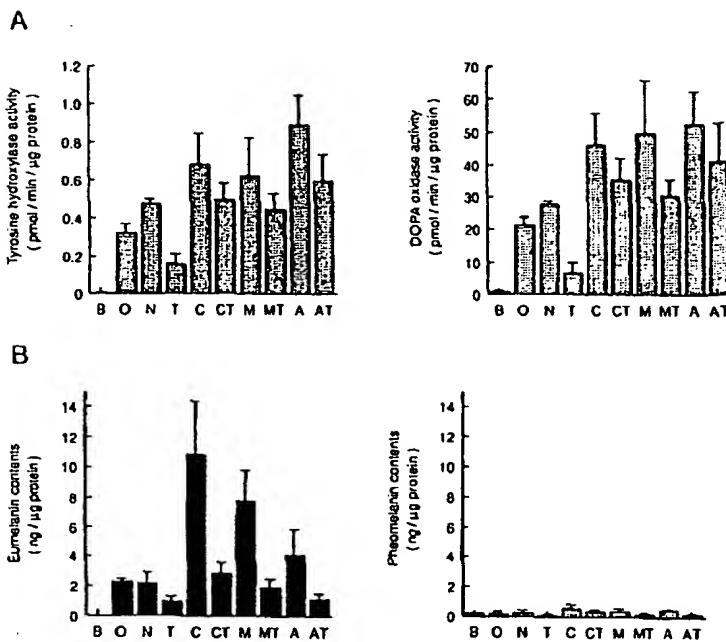
FIG. 2. Effects of the reagents on tyrosine hydroxylase and DOPA oxidase activities (A) and eumelanin and pheomelanin contents (B). Cells (1 × 10⁵) were seeded in 90-mm plates and the reagents were added to the culture medium. The cells were harvested after 48 h of culture, except that O was harvested at 0 hours (start point), and tyrosine hydroxylase and DOPA oxidase activities and eumelanin and pheomelanin contents were measured. The specifications of the treatments are: B, BALB/c 3T3 fibroblast cells (negative control); O, 0 hours (start point); N, no treatment; T, TPA (1.6×10^{-7} M); C, cholera toxin (10^{-9} M); CT, cholera toxin (10^{-9} M) + TPA (1.6×10^{-7} M); M, α-MSH (10^{-6} M); MT, α-MSH (10^{-6} M) + TPA (1.6×10^{-7} M); A, dbcAMP (0.5 mM); AT, dbcAMP (0.5 mM) + TPA (1.6×10^{-7} M). All of the values represent mean values and the bars represent standard deviations (n=4). The significant differences are as follows: tyrosine hydroxylase activity—T, $P < 0.01$; C, $P < 0.05$; A, $P < 0.01$; DOPA oxidase activity—T, $P < 0.01$; C, $P < 0.05$; M, $P < 0.05$; A, $P < 0.01$; eumelanin contents—T, $P < 0.05$; C, $P < 0.01$; M, $P < 0.01$; respectively, compared with N, t test.

shows the time courses of the mRNA accumulations in the cells treated with melanotropic reagents. At all times examined, the changes in the mRNA levels of tyrosinase and TRP-1 were parallel. Cholera toxin elevated mRNA levels of tyrosinase and TRP-1 as early as 6 h after the treatment, but TPA did not show an obvious reduction in mRNA levels until 48 h after the treatment. Thus, it can be said that tyrosinase and TRP-1 genes are activated coordinately by the melanogenic reagents and are inactivated by TPA, although the effects of cholera toxin and α-MSH are rapid and that of TPA occurs after a long lag period.

From the results reported here, we conclude that eumelanin biosynthesis in mouse B16-F1 melanoma cells is regulated by the coordinate expression of the genes for tyrosinase and TRP-1, and that this expression is regulated, if not entirely, at least to a great extent, through cAMP-dependent protein kinase and protein kinase C.

DISCUSSION

Many studies of the effects of melanotropic reagents on pigment cells and their signal transduction pathways

to melanogenesis have been reported. α-MSH and cholera toxin have been shown to stimulate melanogenesis by cAMP accumulation through α-MSH receptors and ADP ribosylation of Gsα, respectively [22, 25–27]. DbcAMP is also known to stimulate melanogenesis [23, 30]. In some melanoma cell lines the elevation of cAMP levels does not always stimulate melanogenesis [27]; however, in most melanoma cell lines, including B16-F1 cells, the accumulation of cAMP correlates with an increase in tyrosinase activity and melanin production [22, 27]. TPA is known to activate protein kinase C [28], and it is reported that this reagent affects melanogenesis. In various melanocyte and melanoma cell lines, TPA shows different effects, in some cases exerting an opposite effect on melanogenesis [24, 26, 30]. It is known that long treatment with TPA suppresses protein kinase C by down-regulation [31]. In various tissues, signal transduction pathways of cAMP-dependent protein kinase and protein kinase C exert positive or negative effects on each other [28]. The cross-talk of signal transduction pathways may occur frequently in many kinds of cells. In mouse B16-F1 melanoma cells, we showed that cholera toxin, α-MSH, and dbcAMP
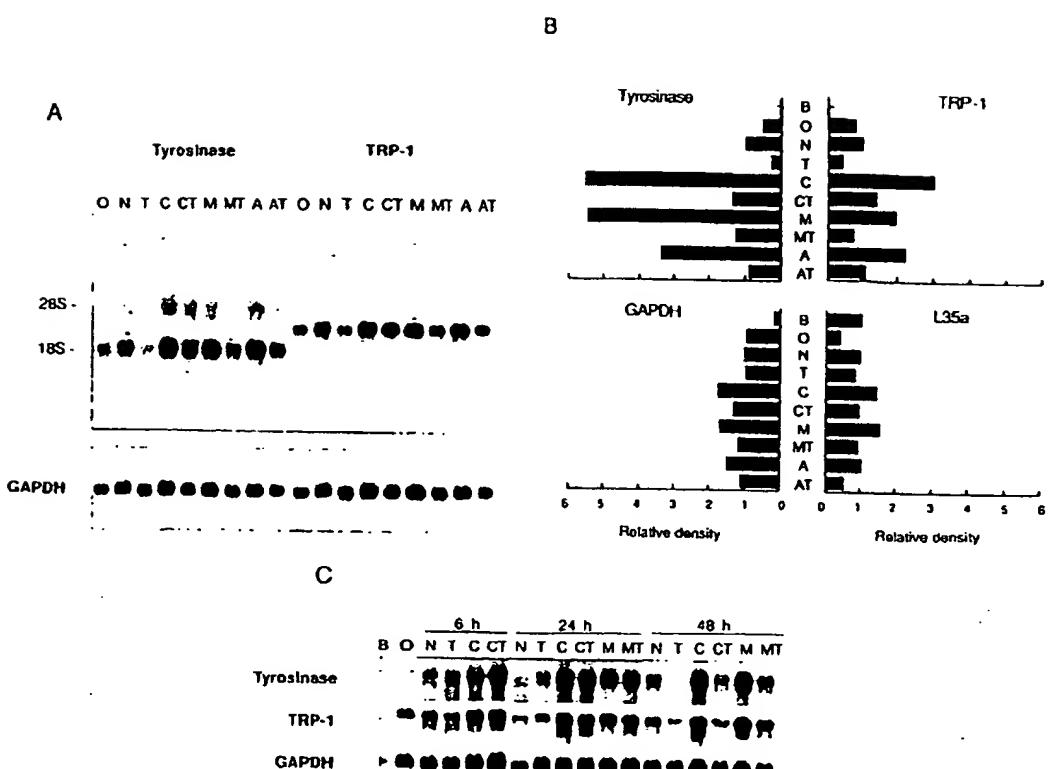
FIG. 3. Northern blot analysis of the mRNA levels of tyrosinase and TRP-1. (A) RNAs were isolated from the cells treated under the same conditions as described in the legend to Fig. 2. Each 7 μg of total RNA was size-fractionated and hybridized with the tyrosinase, TRP-1, and GAPDH probes. These probes were sequentially hybridized using the same RNA-blotted filter after the previously hybridized probes were removed. (C) RNA was isolated 6, 24, and 48 h after the treatment. The other conditions were the same. (B) Densitometric measurement of specific RNA bands of the Northern blot analysis presented in (A). The relative densities of each band are represented in comparison with lane N. The density of lane N is represented as 1. The values represent means of three experiments.

elevated cAMP levels and stimulated melanogenesis. On the other hand, TPA inhibited melanogenesis without having a significant effect on cAMP levels. Based on these results, it can be said that melanogenesis in these cells is enhanced by intracellular cAMP, probably through the activation of cAMP-dependent protein kinase, and is repressed by TPA without suppressing cAMP levels, presumably through protein kinase C. Nevertheless, it is possible that other signal transduction pathways and their cross-talk may be involved in melanogenesis in these cells.

The function of TRP-1 protein is not so well established. It is reported that TRP-1 has no tyrosine hydroxylase activity, no DOPA oxidase activity, no DOPAchrome tautomerase activity, and no 5,6-dihydroxyindole oxidase activity [17]. On the other hand, there is evidence that it has catalase activity [32] or tyrosinase-like activity [33]. Very recently, it was suggested that TRP-1 can stabilize tyrosinase proteins and that it has DHICA oxidase activity (K. Tsukamoto and V. J. Hear-

ing, presented at the 7th Annual Meeting of Japanese Society for Pigment Cell Research). The TRP-1 gene has been mapped to the mouse b locus, and it has been found to rescue the phenotypic change of the brown mutant melanocyte to a black one [34]. Therefore, it is certain that TRP-1 plays an important role in eumelanin biosynthesis.

In mouse melanoma cells treated with α-MSH, dbcAMP, and TPA, the mRNA levels of tyrosinase change in parallel with tyrosinase activities [25, 26]. Using the specific monoclonal antibody, it was shown that the protein content of TRP-1 changed almost in parallel with tyrosinase activity in human melanocytes treated with dbcAMP [30]. In this paper, we showed that the mRNA level of TRP-1 was coordinately regulated with that of tyrosinase by various melanogenic reagents and was reduced coordinately by TPA in mouse B16-F1 melanoma cells. It may not be totally applicable to all pigment cell lines because of their phenotypic differences. However, the coordinate expressions of tyrosinase and

TRP-1 genes are reasonable and purpose-directed for effective regulation of melanogenesis, because eumelanin is produced through sequential reactions regulated by the amounts and activities of some key proteins, including tyrosinase and TRP-1. At present, we do not know whether the change in mRNA levels of tyrosinase and TRP-1 is caused by a change in transcriptional activation or in mRNA stabilities. However, it is certain that transcriptional regulation plays a major role in the change in mRNA levels, which is the initial and most important step of gene expression [35]. Eventually it is supposed that the coordinate transcriptional regulation of tyrosinase and TRP-1 genes causes the coordinate change in the protein contents and activities and finally causes the effective regulation of melanogenesis.

The transcriptional regulation of tyrosinase and TRP-1 genes has been the focus of many studies. Despite the similarity of their protein structures, the gene structures and promoter sequences of tyrosinase and TRP-1 are quite different [36]. The mouse tyrosinase gene has both TATA and CCAAT boxes, which are core elements found in most tissue-specific genes [37]. However, the mouse TRP-1 gene does not have obvious sequences such as TATA and CCAAT box motifs, although it does have an inverted CCAAT sequence on the opposite strand and a sequence similar to a TATA box [36, 39]. The promoter of the tyrosinase gene has putative cAMP-responsive and TPA-responsive elements (CRE, TRE, and AP-2 binding element), although whether they are functional is not evident [26, 37]. In the promoter region of the TRP-1 gene, there are no CRE, TRE, and AP-2 binding consensus sequences [36]. How cAMP-dependent protein kinase and protein kinase C coordinately regulate the expression of the tyrosinase and TRP-1 genes remains to be elucidated.

The 270-bp promoter region containing the transcription start site of the mouse tyrosinase gene [38] and the regions encompassing nucleotide residues −38/+154 [39] and −44/+107 [40] of the mouse TRP-1 gene have been shown to be sufficient for pigment cell-specific expression of these genes. In these promoter regions, a common consensus 11-bp sequence, AGTCATGT-GCT, was found [36, 39, 40]. The sequence is termed an M-box, and a specific protein (M-box-binding factor 1) binds to the sequence and enhances the transcriptional activity of the TRP-1 gene [40]. It is certain that this consensus sequence plays an important role in melanocyte-specific expression. Surprisingly, however, this factor is not specific to melanocyte, since it was also detected in extracts from N1H3T3 fibroblast cells [40]. Further study of the transcriptional regulatory mechanism is awaited to understand the coordinate expression of the melanocyte-specific genes.

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KUZUMAKI ET AL.

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BIOCHEMISTRY OF MELANIN FORMATION

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ELANIN PIGMENTATION has aroused the curiosity and attention of man since the beginning of recorded history. This interest stems in part from the social, protective and cosmetic significance of pigmentation. In addition, much interest has arisen concerning the possible role of melanin formation in the development of the melanoma which has been described as having the most sinister reputation of malignant tumors (1). As Ewing (2) has stated, "The variations in pigment offer a very delicate indication of the functional activity of the cells and a unique opportunity to trace the relation between the functional activity and the growth capacity of the tumor cells." As a result, this subject has attracted the attention of workers in many fields (3), for example, the cytologist in the study of the melanin granules; the geneticist, the anthropologist and the biologist in a study of the relationship of heredity, environment and nutrition to the development of pigment; and the organic and biologic chemists in the study of the chemical mechanism underlying melanin formation.

In the past quarter of a century several comprehensive reviews on the mechanism of melanin formation have appeared (4-11), but in only a few of these has there been any consideration of the biochemical aspects of this problem. No attempt was made to connect the processes of melanin formation operating in various species. Different mechanisms were proposed for humans, lower animals and plants.

Investigation of the biochemistry of melanin formation in plants, insects and marine animals had shown that the enzyme *tyrosinase* catalyzes the oxidation of L-tyrosine to dihydroxyphenyl L-alanine (dopa) and then the oxidation of dopa to melanin.

$$\text{Tyrosine} \xrightarrow{\text{Tyrosinase}} \text{Dopa} \longrightarrow \text{(Intermediates)} \longrightarrow \text{Melanin}$$
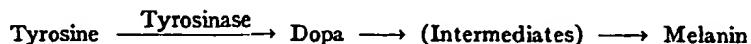
Until recently, however, the presence of tyrosinase in mammalian tissue had not been conclusively demonstrated, and it was believed that melanin in mammalian tissue is formed by a mechanism different from that operating in other species. Histochemical evidence indicated that mammalian skin contains an enzyme, *dopa-oxidase*, which catalyzes the oxidation of dopa, but not tyrosine, to melanin.

$$\text{Dopa} \xrightarrow{\text{Dopa Oxidase}} \text{(Intermediates)} \longrightarrow \text{Melanin}$$

Largely as a result of these beliefs, two separate hypotheses of melanogenesis evolved. Melanin formation in insects and plants was associated with tyrosinase, while melanin production in mammalian skin was associated with dopa-oxidase.

In the present review, it is shown on the basis of recent evidence that these separate concepts can now be merged into a single hypothesis to account for melanin formation in man, lower animals, insects and plants. The properties of the enzymes concerned with melanin formation and the mechanisms of their reactions are considered primarily. Attempts will be made when possible to correlate these properties and reaction mechanisms with normal and pathologic melanin pigmentation.

In view of the widespread occurrence of melanin pigment in nature and the keen interest shown in the problem of melanogenesis, it may seem surprising that there has been so much delay in clarifying the mechanism of melanin formation. Several obstacles have impeded progress in research on melanogenesis. Firstly, melanin (a darkly colored polymerized product of the oxidation of *o*-dihydroxyphenyl compounds, such as dopa, epinephrine, catechol) is of unknown chemical composition. Secondly, it is difficult to purify melanin and as a result, different chemical compositions have been reported by different investigators. Thirdly, epidermal extracts could not be prepared which conclusively demonstrated enzymatic activity toward tyrosine and dopa. This was due either to the small quantity of enzyme in the skin or to the fact that extracts made by grinding and homogenizing skin had not been satisfactory. Finally, the formation of melanin from its precursors is a complex process involving several steps, and the rates of the reactions depend on many surrounding chemical and physical factors.

HISTOCHEMICAL STUDIES

As early as 1901 v. Fürth (12) advanced the hypothesis that melanin formation is the result of the action of an intracellular oxidase on aromatic or chromogen groups in certain protein molecules. Bloch, a Swiss dermatologist, stimulated by this hypothesis attempted to prove it by experimental methods (4). His approach was directed by two clinical observations. First, melanin pigmentation is a prominent feature of Addison's disease, a disorder resulting from hypofunction of the adrenal glands. The possibility arose that there might be some relationship, albeit a paradoxical one, between the increased pigmentation and the metabolism of epinephrine-like substances. Second, in cases of metastatic melanoma with melanuria, the urine contains significant amounts of catechol derivatives. These facts suggested to Bloch a chemical similarity between the precursor of melanin and the compounds epinephrine and catechol. He therefore selected a naturally occurring amino acid, 3,4-dihydroxyphenyl-L-alanine[2] (which he abbreviated as *dopa*, using the initial letters of its German name, *di-oxy-phenyl-alanine*) as the substrate for histochemical studies.

Bloch immersed frozen sections of pigmented human skin in a 1:1,000 solution of dopa buffered to pH 7.3 to 7.4. He noted that after 24 hours at room temperature melanin granules were deposited in the cytoplasm of cells located in the basal layer

[2] Dopa is found in the wing coverings of cockchafers *Melolontha melolontha* L. and *M. hippocastani* F. (13), and in the cocoons of one of the Saturniidae, *Samia cecropia* L. (14). It also occurs in *Vicia faba* (15) and the Georgia velvet bean, *Stisolobium deeringianum* (16).

of the epidermis. These specialized cells, which Bloch called melanoblasts, are located
at the epidermo-dermal junction and were considered by Bloch to be the natural site
of melanin formation. The intensity of the response, moreover, corresponded to the
known capacity of the skin to form melanin. Melanin deposition was most intense
in the areas of the skin capable of heavy pigmentation such as the basal layer of the
epidermis, the germinal layer and cells of the hair matrix, and the cells of pigmented
nevi. Melanin deposition did not occur in albino skin and in the skin of patients with
vitiligo, a skin disease characterized by localized areas of complete loss of melanin
pigment. Bloch (4), therefore, concluded that the 'dopa reaction' is a reliable indica-
tor of the capacity of cells to form pigment.

The origin of the melanoblast has been a subject of controversy for many years. Bloch (4),
Peck (17) and others have supported the thesis that the melanoblasts are derived from ordinary
palisade basal cells under certain types of stimuli. Masson (18) and Becker (19), however, believe
that the melanoblasts are of neuro-epithelial origin. In recent years several groups of investigators
have carried out embryological studies in amphibia (20, 21), birds (22–24) and mice (25–27) and
have shown conclusively that the melanoblasts are derived from the neural crest region.

Further investigation provided evidence that the catalytic effect of certain cells
on the oxidation of dopa to melanin is due to the presence of an enzyme, which Bloch
called *dopa-oxidase*. The evidence for the enzymatic nature of the dopa reaction
included the following: *1)* The reaction did not occur after the tissue had been heated
to 100°C.; *2)* a definite p_H range was required, the optimal p_H being 7.35; *3)* the
reaction was completely inhibited by M/2,000 hydrogen sulfide or M/500 potassium
cyanide, which are known inhibitors of some enzymes; *4)* only the levorotatory
form of dopa was catalytically oxidized by the cells.[1]

To substantiate further the enzyme hypothesis Bloch and Schaaf (28) prepared
extracts of skin from newborn rabbits and demonstrated by visual colorimetric
methods the presence of a heat-labile, cyanide-sensitive catalyst capable of accelerat-
ing the formation of melanin from dopa. Extracts of albino skin under the same con-
ditions were ineffective in catalyzing the oxidation of dopa. Although Bloch's tech-
nics for measuring enzymatic activity were not detailed his data provided convincing
evidence that the catalytic effect of certain epidermal cells on the oxidation of dopa
is due to the presence of an enzyme.

The presence of dopa-oxidase in normal human or animal skin has not been con-
clusively demonstrated by enzymatic technics such as the manometric measure-
ment of oxygen uptake by skin slices or extracts in the presence of dopa. Although
this has been attempted by several investigators, none has been successful because
of the small quantity of enzyme present in the skin as well as the difficulty of pre-
paring skin extracts and homogenates suitable for measuring enzymatic activity.
It is also possible that in preparing extracts of skin the investigators neglected to
take into account the possibility of a naturally occurring inhibitor which could mask
the presence of the enzyme. Recently, Rothman and co-workers (29) succeeded in

[1] Many other related compounds, such as D-dopa, tyrosine, phenylalanine, *p*-hydroxyphenyl-
pyruvic acid, homogentisic acid, quinol, pyrogallol, catechol, protocatechuic acid, 3,4-dihydroxy-
phenylacetic acid, epinephrine, trihydroxyphenylalanine, glycyl-dopa, pyrrole and tryptophane,
did not form melanin granules after incubation with frozen sections of skin.

demonstrating the presence in human epidermis of a water-soluble, dialyzable, heat-stable inhibitor of plant tyrosinase. This inhibitory principle was counteracted by iodoacetamide and another powerful sulfhydryl inhibitor, *p*-chloromercuribenzoic acid. Later work (30) showed a direct relationship between the sulfhydryl group concentration and the inhibitory power of the epidermal extracts.

Bloch's histochemical studies with dopa, which have been amply confirmed, have not been generally accepted as a complete explanation for the mechanism of melanogenesis since dopa has never been demonstrated in mammalian tissue. Raper (31) was able to isolate dopa in crystalline form as the first oxidation product resulting from the action of tyrosinase obtained from mealworms (*Tenebrio molitor*) on tyrosine. This strongly suggested that the reactions in the formation of melanin that are subsequent to dopa production are the same whether the initial substrate is tyrosine or dopa and emphasized the gap in the knowledge of the mechanism of the conversion of tyrosine to dopa in various tissues. Raper (10) provided conclusive evidence that tyrosine, a naturally occurring amino acid present in all tissues including skin, is the initial substrate in the formation of melanin by plant and insect tyrosinase. As stated previously, Bloch's dopa-oxidase reaction could not be obtained after incubation of the frozen skin sections with tyrosine under the same conditions in which dopa was effective. Since it was generally felt that tyrosine is the natural or physiologic substrate in the enzymatic formation of melanin by mammalian tissues as well as by plants and insect tissues, and since the reaction whereby tyrosine is converted to dopa in mammalian cells was not known, the mechanism of melanin formation in mammals remained a puzzling problem for many years.

Much investigation remains to be done on this problem. Bloch's qualitative studies might well be extended by use of present-day microchemical methods for quantitative determinations and histologic localizations of small quantities of enzymes. In addition, through the use of C^{14}-labeled tyrosine it may be possible to show conclusively whether or not tyrosine is the natural substrate involved in melanin formation by melanoblasts.

BIOCHEMICAL STUDIES

Melanin Formation in Lower Animals—the Tyrosinase Concept

The enzymatic nature of the melanin-producing reactions was first satisfactorily investigated in plants and fungi. In 1895 Bourquelot and Bertrand (32) reported that a substance present in the mushroom, *Russula nigricans*, was converted into a black pigment. This substance, later shown to be tyrosine, was acted on by an enzyme in the fungus which was named *tyrosinase* (33). Since that time tyrosinase has been found in a wide variety of plant and animal tissues (34–36) and a vast literature has accumulated on this subject.

Working with tyrosinase obtained from plants and mealworms, Raper (10) was able to determine many of the reaction mechanisms whereby tyrosine is converted into melanin. He showed that in the presence of tyrosinase and oxygen, tyrosine is first oxidized to dopa, and the dopa is then oxidized to dopa-quinone. Dopa-quinone is converted to an indole derivative which, after undergoing several

reactions, polymerizes to form the pigment, melanin. The details of the chemical reactions concerned in this process (fig. 1) will be considered later.

The literature contains several early reports on the occurrence of tyrosinase in mammalian tissue. In 1903, Gessard (37) found that extracts from a horse melanoma were able to catalyze the conversion of tyrosine to melanin. This was confirmed by De Coulon in 1920 (38). In 1907, Alsberg (39) prepared from a human melanoma an extract which catalyzed the formation of black pigment from catechol and possibly from tyrosine. In the following year, Neuberg (40) showed that dilute extracts from a human melanoma accelerated pigment formation from tyramine and epineph-
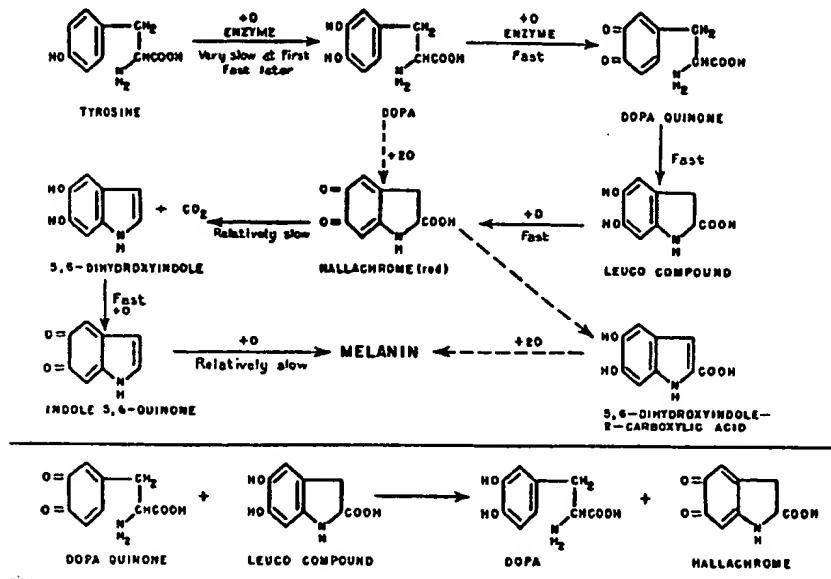
Fig. 1. ENZYMATIC oxidation of tyrosine to melanin.

rine but not from tyrosine. Winternitz reported in 1919 (41) that an enzyme present in the uvea of the hog and the black cutaneous tissue of the horse could catalyze the darkening of tyrosine.

Melanin Formation in Mammals—the Dopa-Oxidase Concept

Despite the early widespread beliefs that tyrosinase occurred in mammalian tissue, plus experimental evidence in accord with these beliefs, skepticism arose concerning the presence of the enzyme in such tissue. For example, the work of Durham (42) on tyrosinase in fetal rabbit skin could not be confirmed (43). In addition, no one was able to demonstrate tyrosinase activity in normal pigmented mammalian tissue. Finally, Bloch (4) as discussed previously, showed that an enzyme present in mammalian skin could catalyze the oxidation of dopa but not tyrosine to melanin. Largely as a result of this work, the concept arose that *dopa-oxidase*, but not *tyrosin-*

ase, was present in mammalian tissue. Although Bloch's work did not pass unchallenged, it remained the most acceptable until recently.

Hogeboom and Adams in 1942 (44), Greenstein and co-workers in 1944 (45, 46) and Lerner, Fitzpatrick, Calkins and Summerson in 1949 (47) showed conclusively that extracts from mouse, human and horse melanomas contain both tyrosinase and dopa-oxidase activities and that these activities are similar to those found in extracts from plants and lower animals. Calkins (48) demonstrated that extracts from normal beef ciliary bodies possess tyrosinase and dopa-oxidase activities. In view of this experimental evidence of the presence of tyrosinase in mammalian tissue, it became necessary to modify the hypotheses of pigmentation which had evolved from Bloch's dopa-oxidase studies.

Chemical Reactions in the Conversion of Tyrosine to Melanin

The reactions involved in the enzymatic oxidation of tyrosine to melanin are shown in figure 1. In the presence of tyrosinase and molecular oxygen, tyrosine is oxidized to dopa. This reaction is usually slow at the onset, but after an induction period it becomes very fast. The conversion of tyrosine to dopa is not a reversible reaction. Dopa formed in the first reaction is oxidized enzymatically by a reversible reaction to dopa-quinone. Further stages of the reaction proceed rapidly in the absence of the enzyme although the reaction rates are increased in the presence of the enzyme. Dopa-quinone undergoes a spontaneous irreversible and rapid intramolecular change in which the nitrogen of the side chain attaches itself to the 6-position of the benzene nucleus with the formation of 5,6-dihydroxydihydroindole-2-carboxylic acid (leuco compound). The leuco compound is readily oxidized by a reversible reaction to the corresponding quinone (hallachrome). Hallachrome[4] is a red substance, and it is the first visible product formed in the reactions. Under physiologic conditions hallachrome decarboxylates and undergoes a rearrangement to form 5,6-dihydroxyindole. The indole compound is rapidly oxidized to the corresponding quinone which has a purple color. The quinone then polymerizes to melanin with the consumption of approximately one atom of oxygen. Relatively little is known of the mechanism of this polymerization (49, 50). If the intramolecular rearrangement undergone by hallachrome is quickened by sulfurous acid, no decarboxylation occurs and 5,6-dihydroxyindole-2-carboxylic acid is formed. This latter substance is readily converted to a melanin substance. In the series of reactions shown in figure 1, possible alternate mechanisms are described by broken arrows. Much of the knowledge of the chemical reactions which take place in the enzymatic oxidation of tyrosine has been obtained through the brilliant work of Raper and his co-worker (10, 52a) with potato and mealworm (*Tenebrio molitor*) tyrosinase. They were able to show that the following three substances were formed during the tyrosine-

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[4] Hallachrome occurs naturally in the polychaete worm, *Halla parthenopaia* (51). Friedheim (52) found that this red substance could accelerate oxygen consumption by erythrocytes and serve as a hydrogen acceptor for xanthine oxidase and succinic dehydrogenase. He suggested that hallachrome may play a role in cellular respiration.

tyrosinase reaction:
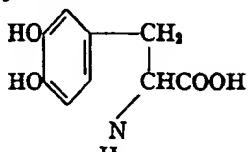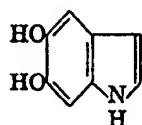
I, Dopa   II, 5,6-Dihydroxyindole   III, 5,6-Dihydroxyin-
                                             dole-2-Carboxylic Acid
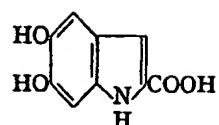
When the enzyme is allowed to act on dopa, *substances II* and *III* are formed. This suggests that dopa is probably the first compound formed in the oxidation of tyrosine.

Dopa formed from tyrosine appears to be oxidized to dopa-quinone. The following facts make this plausible: *1*) *o*-dihydroxyphenyl compounds are readily oxidized to the corresponding orthoquinones; *2*) substances which react with orthoquinones inhibit melanin formation in the dopa-tyrosinase reaction (53); *3*) plant tyrosinase has been shown to catalyze the oxidation of catechol to orthobenzoquinone (54). The oxidation of dopa to dopa-quinone would appear to be a similar reaction.

Raper (55) also showed that a red substance (hallachrome) was formed in the oxidation of dopa. This red substance could be reduced to 5,6-dihydroxydihydroindole-2-carboxylic acid (leuco compound) (52a), and the leuco compound so produced readily oxidized back to the red substance. From this it seems likely that dopaquinone undergoes an intramolecular change, the nitrogen of the side chain attaching itself to the 6-position of the benzene nucleus with the resultant formation of the leuco compound.

Since the indole *compounds II* and *III* could be formed from the oxidation of the hallachrome, and since hallachrome was formed from the leuco compound, it was believed that the hallachrome was simply the quinone of the leuco compound and therefore should have the following structure:
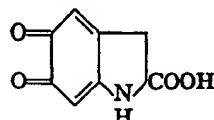
Hallachrome

Mason has presented spectrophotometric evidence to support this view (49).

Under normal conditions in the enzymatic oxidation of tyrosine, hallachrome is converted, by decarboxylation and rearrangement, to 5,6-dihydroxyindole. Spectrophotometric data (49) indicate that the 5,6-dihydroxyindole is then rapidly oxidized to the corresponding quinone and the quinone then polymerizes to melanin.

It should be pointed out that the reactions given in figure 1 merely represent the over-all scheme by which tyrosine is converted to melanin. Actually, many more reactions probably occur, such as those involving the formation of semiqui-

nones. The semiquinones may then be oxidized to quinones or undergo rearrangements in accordance with the general picture given in the diagram.

As previously stated, enzyme action is definitely required for the first reaction, the oxidation of tyrosine to dopa. Under physiologic conditions, that is, pH 7 to 7.4, the rate of oxidation of dopa to dopa-quinone is fairly rapid without the enzyme but is increased appreciably in the presence of the enzyme. The subsequent reactions shown in the diagram take place rapidly without the enzyme, but even in these cases there is evidence that the presence of tyrosinase will increase the rate of reaction (49).

In addition to the series of reactions discussed above, there is an important interplay of reactions occurring during the conversion of tyrosine to melanin as shown in the lower part of figure 1. Using mealworm tyrosinase, Evans and Raper (52a) found that in a tyrosine-tyrosinase reaction which has proceeded for two to five hours, dopa can be isolated in yields varying from 10 to 20 per cent of the actual tyrosine oxidized, in spite of the fact that this tyrosinase can oxidize dopa more readily than tyrosine. Since the conversion of dopa-quinone to leuco compound does not appear to be a reversible reaction, it seems reasonable to explain the accumulation of dopa on the basis of the presence of some reducing agent or system which reduces the dopa-quinone back to dopa. The oxidation-reduction potentials of the systems concerned in melanin formation in so far as they have been investigated (56) are as follows:

$$\text{Dopa} \rightleftharpoons \text{Dopa-Quinone}$$
$$E_0 = + 0.511 \text{ V at } pH \text{ 4.6}$$

$$\text{Leuco Compound} \rightleftharpoons \text{Hallachrome}$$
$$E_0 = + 0.170 \text{ V at } pH \text{ 46.}$$
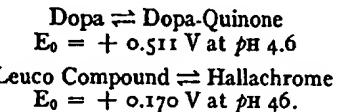
From these data and from the observation that addition of leuco compound to a tyrosine-tyrosinase system increases the accumulation of dopa, it seems likely that the interplay of reactions shown in figure 1 occurs in the tyrosine-tyrosinase reaction. The importance of this reaction will be discussed later. It is possible that interactions involving substances such as dopa-quinone and 5,6-dihydroxyindole also occur. These reactions, if present, could play important roles in regulating the rate of melanin formation.

Induction Period in the Oxidation of Tyrosine

There are several points in the oxidation of tyrosine that are of great importance and interest. When tyrosine and tyrosinase are allowed to react in the presence of oxygen, there is often a lag period before oxidation of tyrosine begins. This lag interval is referred to as the *induction period* (47). Small amounts of dopa are very effective in shortening the induction period in the tyrosine-tyrosinase reaction (fig. 2). If the induction period is defined as the intercept on the time axis of an extension of the slope of the oxidation curve when oxidation is proceeding maximally, there is for mammalian tyrosinase a linear relationship between the negative logarithm of the dopa concentration and the induction period (47).

Recent studies (57, 58) with mouse melanoma tyrosinase indicate that compounds related structurally to dopa, for example, epinephrine, catechol, and so

forth, can shorten the induction period, but not nearly as effectively as dopa. On an equimolar basis, DL-dopa is about 75 per cent as effective as is L-dopa in shortening the induction period. For mammalian tyrosinase, dopa is a fairly specific catalyst regulating the induction period.

When dopa is used as the substrate instead of tyrosine there is no induction period. Although dopa is required to catalyze the enzymatic oxidation of tyrosine, dopa itself is rapidly oxidized. Hence the rate of tyrosine oxidation is dependent on the rate of dopa oxidation.
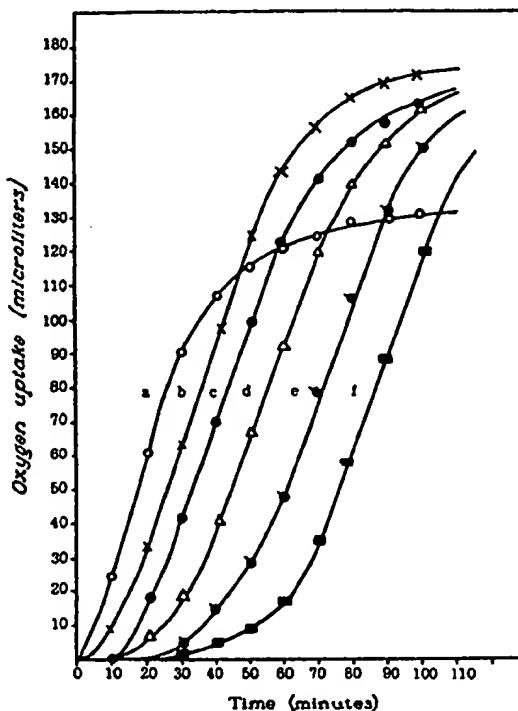
Fig. 2. EFFECT OF DOPA on induction period in enzymatic oxidation of tyrosine by mouse melanoma preparation at *p*H 6.8 and 38°C. *a* 0.5 mg. dopa; *b* 0.1 mg. dopa plus 0.4 mg. tyrosine; *c* 0.05 mg. dopa plus 0.45 mg. tyrosine; *d* 0.01 mg. dopa plus 0.49 mg. tyrosine; *e* 0.001 mg. dopa plus 0.50 mg. tyrosine; *f* 0.5 mg. tyrosine. (*J. Biol. Chem.* 178: 192, 1949).

It can be seen from figure 2 that after the induction period is over the rates of oxidation of tyrosine and dopa are practically identical. This observation at first glance seems difficult to understand, because one might expect that as the dopa formed from tyrosine is oxidized, the rate of reaction would diminish. The explanation of this finding is that dopa is not only oxidized in the reaction but also reformed during the oxidation, as discussed previously (fig. 1, lower part). Consequently, a significant amount of dopa is always available to shorten the induction period.

From what has been described it can be seen that the presence in the tyrosine-

tyrosinase reaction of any substance which is capable of reducing dopa-quinone back to dopa and thereby causes an accumulation of dopa should shorten the induction period. This is indeed the case. Either ascorbic acid or hydroquinone[5] shortens the induction period in tyrosine oxidation. Melanin, however, is not produced until all the ascorbic acid or hydroquinone has been oxidized. It is possible that the compounds related to dopa shorten the induction period by acting merely as non-specific reducing agents which influence the induction period as described previously.

It should be pointed out that it is well known that *o*-dihydroxyphenyl compounds shorten the induction period in the oxidation of monophenols with tyrosinase obtained from plants and lower animals. Most of the work on this subject, however, has been done with catechol, phenol and *p*-cresol and not with dopa and tyrosine (54). No emphasis has been placed on structural specificity required by the orthodihydroxyphenyl compound. Actually, this structural specificity could play an important role in melanin formation in nature.

The mechanism by which substances such as dopa regulate the induction period of the tyrosine-tyrosinase reaction is not fully understood. Recently, the authors carried out potentiometric measurements using the platinum electrode on different solutions of tyrosine, dopa and tyrosinase obtained from the Harding-Passey melanoma (59). Solutions of tyrosine in 0.1 molar potassium phosphate buffer were found to be at a much higher potential than similar equimolar solutions of dopa. On the addition of tyrosinase to the tyrosine solution the potential began to fall. When the potential had fallen to a value approximately equal to that of the buffered dopa solution, oxygen uptake commenced. No appreciable oxygen uptake could be detected before the potential fell. As the oxidation proceeded and the tyrosine was converted to melanin, the potential began to rise; and when the oxidation was complete, the original potential was re-established. When the tyrosinase was added to the dopa solutions, oxidation began immediately and the potential began to rise. At the end of the reaction the potential reached a value similar to that obtained in the tyrosine-tyrosinase system. Small amounts of dopa added to the tyrosine solutions brought about an immediate lowering of the redox potential.

If the redox potentials were to influence the induction period, one could predict that increasing the tyrosine concentration of a tyrosine-tyrosinase reaction mixture would prolong the induction period. This was found to be the case. The redox potential of a system is established by the ratio of the quantity of reduced form of a substance to the oxidized form. An increase in tyrosine concentration increases the concentration of the reduced form of the substrate.

While the redox potential of the tyrosinase system may play a role in regulating the induction period (as well as tyrosinase activity in general) it is not the only factor involved. Equimolar quantities of DL-dopa are not as effective as is L-dopa in shortening the induction period, but there is no reason to believe that DL-dopa should establish a redox potential different from that obtained with the same quantity of L-dopa. It appears that dopa is a fairly specific catalyst for the enzymatic oxidation of tyrosine.

Nature of Tyrosinase

Tyrosinase can be obtained from various sources simply by grinding the tissues (for example, potato, fungus, melanotic tumor, and so forth) with an aqueous solution and then centrifuging the mixture at low speeds. The supernatant usually contains the active enzyme (47). Further purification can often be obtained by using ordinary procedures for protein fractionation.

[5] In addition to shortening the induction period by virtue of its reducing properties, hydroquinone also appears to inhibit the enzyme.

There are many qualitative differences among tyrosinases prepared from various sources, but they all have three characteristics in common: *1*) All catalyze the oxidation of tyrosine to melanin (presumably by the series of reactions shown in fig. 1); *2*) the enzymatic reaction with the monohydroxyphenyl compound is catalyzed by some o-dihydroxyphenyl compound (dopa, catechol etc.); *3*) copper is associated with the activity of the enzyme. The first two points have been discussed previously.

Role of copper. Copper has been reported to be an essential part of tyrosinase prepared from mammalian (60), plant (61, 62) and insect tissue (63). Mouse melanoma tyrosinase can be inhibited by reagents that combine with copper (diethyldithiocarbamate, BAL, etc.) (60). This inhibition was reversed by the addition of an excess of cupric ions. Treatment of the enzyme with cyanide followed by dialysis resulted in a decrease of the copper content of the enzyme preparation and a loss of enzymatic activity. Addition of sufficient cupric ions resulted in almost complete restoration of activity. Other metals (iron, cobalt, nickel, magnesium, manganese and zinc) were ineffective in restoring enzymatic activity. Previous experiments by Kubowitz (61, 62) with plant tyrosinase and by Allen and Bodine (63) on grasshopper tyrosinase showed essentially the same results as those obtained with mammalian tyrosinase.

Properties of tyrosinase from different sources. As mentioned previously, there are several properties by which tyrosinase from different sources varies. Tyrosinase prepared from plant tissue can usually be obtained in colloidal solution. Tyrosinase obtained from mammalian tissue however is retained on ultramicroscopic cytoplasmic particles. As yet no method has been found by which the active enzyme can be separated from the particles. Hence, it must be realized that, when working with aqueous mammalian tyrosinase preparations, one is dealing with a suspension of particles which have molecular weights greater than those of most proteins.

Tyrosinase from plants and lower animals appears to be less specific in its action than is mammalian tyrosinase. Some plant tyrosinases are able to catalyze the oxidation of many phenol derivatives and orthodihydroxyphenyl compounds at a greater rate than the oxidation of tyrosine and dopa. With mammalian tyrosinase, on the other hand, tyrosine and dopa are oxidized at a much greater rate than any other substance related structurally to these amino acids (57, 58).

Sizer (64, 65), working with mushroom tyrosinase, reported some interesting findings on the oxidation of tyrosine present in the peptide chain of proteins. These findings support the contention that plant tyrosinase acts on combined as well as on free tyrosine. The effect of mammalian tyrosinase on tyrosine in proteins has not been tested; but, since this enzyme cannot catalyze the oxidation of tyrosine in which a hydrogen atom of the amino group is replaced by an acetyl or formyl group (57, 58), it is unlikely that tyrosine which is linked to another amino acid through its amino group could be oxidized by the mammalian enzyme.

Tyrosinase obtained from grasshopper eggs (66–69) occurs as a *protyrosinase*. This enzyme, unlike mammalian tyrosinase, must first be activated before it can exert any catalytic action on tyrosine (or related compounds). The activating factors are usually substances such as distilled water, sodium chloride, detergents, changes in *p*H or temperature.

Stability of tyrosinase. Tyrosinase from different sources varies greatly in its stability toward physical and chemical agents. The following information is available on the stability of a crude particulate suspension of tyrosinase from the Harding-Passey mouse melanoma (47). The enzyme preparations may be kept in solution at 5° C. for two months with no apparent loss of activity. Heating the preparations for ten minutes at 70° C. results in complete inactivation. Lyophilization does not alter enzymatic activity. Dialysis against water at 5° C. has no effect on the enzyme. Preparations may be kept at 5° C. in solutions ranging in pH from 4.7 to 8.0 for 24 hours without loss of enzymatic activity when reactions are later carried out at pH 6.8. Some fungus tyrosinases are inactivated after such treatment. The addition of 0.1 M acetate buffer at pH 4.7 to the mammalian enzyme preparation results in the formation of a precipitate which contains all the active material. The supernatant is inactive.

Plant tyrosinase is inactivated during reactions with various hydroxyphenyl compounds (54). The mouse melanoma tyrosinase referred to above does not appear to be readily inactivated during the reaction. If dopa is added to a reaction mixture which has previously oxidized dopa to melanin, the rate of oxidation is the same as that of the original reaction.

Effect of temperature on reaction rates. In general, the rate of enzymatic oxidation of tyrosine and dopa by preparations from the mouse melanoma increases with an increase in temperature. An increase in temperature also shortens the induction period in the oxidation of tyrosine. The temperature coefficient for the oxidation of dopa is only 1.2 at less than 37° C. (47). Above 37° C. the temperature coefficient increases to 1.7. This variation in the temperature coefficient is further indication that the oxidation of dopa to melanin is not a simple reaction. The biologic significance of the influence of temperature on reaction rates in melanin formation will be discussed later.

Effect of pH on reaction rates. It is difficult to evaluate the effect of pH on the enzymatic oxidation of dopa because dopa is readily oxidized in solutions kept at pH 7.0 or more even in the absence of the enzyme. With mouse melanoma tyrosinase the optimal pH for the oxidation of dopa is about 6.8. At pH 5.0 a marked decrease in the rate of oxidation occurs.

The induction period in the enzymatic oxidation of tyrosine appears to be at a minimum at pH 6.8. At pH values greater than and less than 6.8 the induction period increases. Above pH 8.5 and below pH 5.0 the induction period is prolonged indefinitely.

Effect of substrate concentration on total oxygen uptake. The total oxygen uptake during a reaction with tyrosinase is directly related to the initial concentration of tyrosine (or dopa) in the reaction mixture. If the concentration of substrate (within limits) is increased twofold, the total oxygen uptake is likewise increased twofold. The total amount of oxygen required to oxidize tyrosine and dopa to melanin is difficult to determine with great precision. Most reports (47, 70) indicate that each tyrosine and dopa molecule requires approximately five and four atoms of oxygen, respectively, for conversion to melanin. Variations in the concentration of enzyme affect somewhat the total amount of oxygen consumed in a reaction (71, 71a).

Is tyrosinase one or two enzymes? An important problem in the mechanism of melanin formation is whether tyrosinase is one or two enzymes. The following possibilities exist: *1*) One enzyme, tyrosinase, may be involved in melanin formation. If so, this enzyme possesses two distinct activities. First, it can effect the addition of an OH group to the benzene nucleus of a monohydroxyphenyl compound. Second, it can catalyze the removal of two hydrogen atoms from an *o*-dihydroxyphenyl compound. *2*) Two separate enzymes may be involved in melanin formation with each enzyme possessing a single activity. For example, one enzyme, tyrosinase, could catalyze the addition of an OH group to the benzene nucleus. A second and different enzyme, dopa-oxidase, could catalyze the removal of two hydrogen atoms from an *o*-dihydroxyphenyl compound. *3*) A third possibility to be considered is whether or not tyrosinase may be one enzyme plus an additional factor. These two substances together could possess the two catalytic activities described in the previous paragraphs.

Current evidence supports the view that tyrosinase is a single enzyme with two activities. This concept has been championed by Nelson and Dawson (54) and Mallette (72), whose notable work on plant and mushroom tyrosinase provided the experimental basis for the one-enzyme hypothesis. The following points lend support to this hypothesis: *1*) no tyrosinase preparation yet obtained has been satisfactorily demonstrated to catalyze the oxidation of monohydroxyphenyl compounds, but not that of *o*-dihydroxyphenyl compounds; *2*) the reverse statement is also true; namely, all enzyme preparations that catalyze the oxidation of *o*-dihydroxyphenyl compounds can catalyze the oxidation of monohydroxyphenyl compounds under the proper conditions; *3*) enzyme preparations have been obtained in which the ability to catalyze the oxidation of both the monohydroxyphenyl and *o*-dihydroxyphenyl compounds was proportional to the copper content of the preparation. Mallette and Dawson obtained from mushrooms a purified tyrosinase preparation which was homogeneous electrophoretically and almost homogeneous in the ultracentrifuge. The properties of this highly purified preparation were in accord with the foregoing points.

Recently a single-enzyme hypothesis was proposed to account for melanin formation in mammalian tissue (47). Studies with mammalian tyrosinase obtained from the Harding-Passey mouse melanoma showed that it was not possible to separate tyrosinase and dopa-oxidase activities although fractions with long induction periods in the oxidation of tyrosine could be obtained. These fractions were superficially free of tyrosinase activity; however, they catalyzed the oxidation of tyrosine rapidly and completely in the presence of small amounts of added dopa. For these reasons it was suggested that "the separate terms *tyrosinase* and *dopa-oxidase* be abandoned in favor of the single term *tyrosinase* to describe the enzyme (or enzyme complex) involved in the oxidation of both tyrosine and dopa to melanin." Further support of this concept is to be found in recent work which showed that N-acetyltyrosine and N-formyltyrosine are competitive inhibitors for the dopa-tyrosinase reaction (58). It is possible that dopa and the N-tyrosine derivatives compete for the same active centers on the enzyme molecule.

In the authors' opinion the term 'tyrosinase' is preferable to 'dopa-oxidase,' 'polyphenoloxidase' or 'phenolase.' The only justification for retaining the term dopa-oxidase (or 'dopase') is that

dopa, or some other dihydroxyphenyl compound, may be the initial substrate in the enzymatic formation of melanin because a dihydroxyphenyl compound is required to catalyze the tyrosine-tyrosinase reaction. Important objections to this view are, first, tyrosine is a more abundant natural substrate than dopa in mammalian melanin formation. Several dihydroxyphenyl compounds other than dopa can initiate the tyrosine-tyrosinase reaction; and it is possible that dopa comes, for the most part, only from the enzymatically oxidized tyrosine. Second, since dopa is readily oxidized to melanin in the absence of any specific enzyme, dopa-oxidase is often used as a non specific term. For example, the oxidation of dopa by oxidizing agents in an active cytochrome system is often mistakenly referred to as a dopa-oxidase reaction. For these reasons tyrosinase is a more suitable term.

Since no substance is more active or abundant than tyrosine as a substrate for the enzymatic formation of melanin by mammalian tissue, it is not desirable to use the general terms 'polyphenoloxidase' or 'phenolase.' However, tyrosinase might be considered a type of polyphenoloxidase or phenolase.

The two-enzyme hypothesis is not supported by direct experimental evidence. As yet, no enzyme capable of catalyzing the oxidation of tyrosine and dopa to melanin has been shown to be homogeneous by adequate critical experimental work. Until this is done, the possibility remains that the oxidation of tyrosine and dopa may involve separate enzymes.

The single-enzyme-plus-additional-factors hypothesis was suggested by Keilin and Mann (73). They prepared a purified oxidase from mushrooms, which they claimed was specific in catalyzing the oxidation of a small group of polyphenols. They expressed the belief that the oxidation of monophenols probably requires the presence of an additional factor. This view, with a change in emphasis, fits well with the one-enzyme hypothesis.

In accordance with the foregoing discussion and earlier statements it is suggested that the single term tyrosinase be used to include the separate terms tyrosinase and dopa-oxidase. This concept is illustrated diagrammatically below.
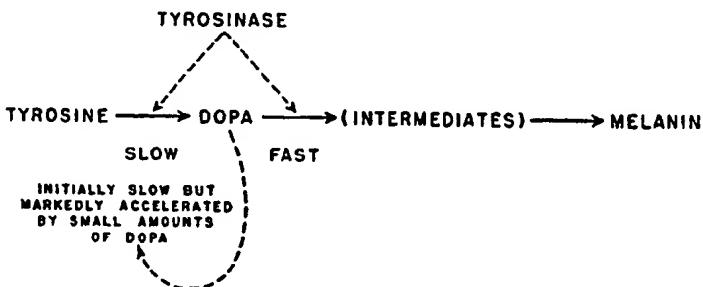
At the time Bloch carried out his important histochemical studies little was known about the optimal conditions for the enzymatic oxidation of tyrosine. This may account for the fact that Bloch, working with mammalian tissue slices, obtained melanin formation from dopa but not from tyrosine.

In some recent histochemical experiments (74) in collaboration with S. William Becker, Jr., we have demonstrated the formation of melanin from tyrosine in human

white skin which had been irradiated with ultraviolet radiant energy for one to five days before excision. Tissue slices cut from the biopsy material were incubated in tyrosine solutions at *p*H 7.1 for 24 to 48 hours. In paraffin sections of this material, there are large dendritic melanoblasts containing melanin granules in their cytoplasm, identical in their morphology with the 'dopa positive' cells obtained by Bloch. The catalytic effect of these cells on the oxidation of tyrosine to melanin is absent when the sections are heated for ten minutes at 100° C. Since tyrosine, in contrast to dopa which readily auto-oxidizes, is a stable amino acid which does not oxidize spontaneously to melanin *in vitro*, it is likely that the melanoblasts of human skin contain an intracellular oxidase, tyrosinase, similar to the enzyme described previously. The enzyme apparently exists in human skin in a partially inhibited state, and can be activated by ultraviolet radiant energy. The mechanism of this activation is not fully understood, but the inactivation of epidermal sulfhydryl groups by the ionizing radiation appears to play an important part.

NATURE OF MELANIN

The word 'melanin' is derived from the Greek *melas*, meaning black. It is used to denote various shades of brown and black pigments found in mammals, insects, plants and marine animals and ·produced *in vitro* by the oxidation of dihydroxyphenyl compounds. These pigments result from the polymerization of the oxidation products of dihydroxyphenyl compounds (dopa, epinephrine, catechol, and so forth) to relatively insoluble substances of high molecular weight. It is amazing that one term *melanin* has been used for many years to describe these natural and synthetic pigments even though there are a variety of melanins and even though no exact definition for the term can be given.

With the aid of the electron microscope Mason and co-workers (75) studied melanin granules from colored human skin; from the ciliary body, choroid, and iris of beef eyes; and from the S91 and Harding-Passey mouse melanomas. They found that the melanin granules were characteristically regular, spheroid particles. The particles appeared as formed elements and not simply as precipitated aggregates.

Because melanin is relatively insoluble in most solvents, it is difficult to isolate and purify from tissue sources. An additional factor making purification difficult is that melanin is often bound to protein in the tissues. Gortner described a melanoprotein present in sheep wool (76), and Greenstein and co-workers (77) have recently described a melanin-containing pseudoglobulin present in the S91 mouse melanoma. From the work of Sizer (64, 65) it appears that melanoproteins can be produced *in vitro* by the action of mushroom tyrosinase on the tyrosine within intact protein molecules.

An approximate composition of melanin from natural and synthetic sources, obtained by averaging the values in the literature, is as follows: carbon 57 per cent, hydrogen 3.5 per cent and nitrogen 9 per cent (35, 78). Oxygen is also present. Varying amounts of sulfur have been reported to occur in some natural melanins, but in some cases the sulfur can be removed (79). In other cases (77) the presence of sulfur seems to be associated with the amino acids of protein which is bound to melanin.

Melanin obtained from the ink sac of the squid or produced by the auto-oxidation of dopa or by the action of tyrosinase on tyrosine can be decolorized from jet black to light tan by sodium hydrosulfite (80, 81) or ascorbic acid (82). The tan-colored melanin produced with the former reducing agent can be changed to black again by the addition of potassium ferricyanide to the reaction mixture.[6] As a result of these findings, Figge (81) suggested that melanin can form a reversible oxidation-reduction system. These interesting findings must be explored further to determine the mechanism of

$$\text{Reduced Melanin (tan)} \rightleftharpoons \text{Oxidized Melanin (black)}$$
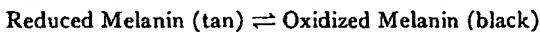
the process and to relate them to biologic systems. It is possible to predict that if there is reduction of melanin *in vivo*, the reduction does not occur at the site of melanin formation at the same time that melanin is being produced.

In 1939, Edwards and Duntley (83) suggested that the term melanoid be used when one is referring to the diffuse melanin pigmentation in the stratum corneum of human skin. They claimed that the corneum from the heel pad of a cadaver had a light-absorption maximum in the visible violet instead of the ultraviolet as does melanin. From this they concluded that the yellow pigment of the heel pad resulted from a disintegration of melanin. More work is needed to establish this point and until this is done the term melanoid should be used only with qualifications.

The various races (for example, white, oriental and Negro) appear superficially to have differently colored skins. All investigative work on this subject however indicates that melanin is the main pigment of human skin, and that the variation in color of skin from different races is due to the variation in quantity of melanin only (84). The spectrophotometric analyses of human epidermis reported by Brunsting and Sheard (85) and by Edwards and Duntley (83) support this view.

There are several ways to classify melanins because the nature and the sources of these pigments vary. In most classifications melanin is divided into two groups: natural and synthetic (86). The natural melanins can be subclassified according to their biologic source, and the synthetic melanins can be subclassified according to their mode of formation.

INHIBITORS OF MELANIN FORMATION

From a consideration of the properties of the enzyme tyrosinase which takes part in melanogenesis, and with a knowledge of the mechanism of the reactions involved in pigment formation it is possible to anticipate methods of stopping the reaction at certain stages. A list of some of the known inhibitors of melanin formation is given in table 1.

Substances That Combine with Copper

Inhibition of tyrosinase results in a decrease in the rates of the tyrosine-tyrosinase and dopa-tyrosinase reactions. Such inhibition can usually be achieved *in vitro* by binding (or removing) the copper ions, which are necessary for tyrosinase action, with substances that form weakly dissociable complexes with copper. Common in-

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[6] However, vigorous oxidation of the black melanin apparently produces a substance which is first red and then colorless.

hibitors are organic sulfur-containing compounds (60, 87–89), hydrogen sulfide (4), carbon monoxide (4, 44) and cyanide ions (4, 44). The organic sulfur-containing compounds which have been used are phenylthiourea, α-naphthylthiourea, diethyl-dithiocarbamate, 2,3-dithiopropanol, cysteine, glutathione, thiouracil, thiourea and phenylthiocarbamide.

TABLE 1. INHIBITORS OF MELANIN FORMATION IN VITRO

I. Substances that combine with copper		*IV. Substances that combine with o-dihydroxy groups(?)*
A. Phenylthiourea[1]	pK[2] = 5.60	
B. Diethyldithiocarbamate	pK = 4.00	A. Sodium molybdate[1]
C. 2,3-Dithiopropanol (BAL)	pK = 3.85	
D. Cysteine	pK = 2.90	*V. Substances that combine with orthoqui-*
E. Glutathione	pK = 2.35	*nones*
F. Thiouracil[1]	pK = 2.05	A. Aniline
G. Thiourea	pK = 1.75	B. Aminotyrosine
		C. *p*-Phenylenediamine
II. Competitive inhibitors		
A. N-Acetyltyrosine	pK = 3.45	*VI. Reducing substances*
B. N-Formyltyrosine	pK = 3.35	A. Ascorbic acid[1]
C. Fluorotyrosine	pK = 2.50	
		VII. Hydroquinones
III. Substances or conditions that prolong the induction period of tyrosine oxidation		A. Hydroquinone[1]
A. 'Tween-20'		B. *p*-Benzylhydroquinone[1]
B. Changes in *p*H		

[1] Also inhibits melanin formation *in vivo*.
[2] pK is used to indicate the negative logarithm of the concentration of inhibitor that produces 50 per cent inhibition of the dopa-tyrosinase (Harding-Passey mouse melanoma) reaction.

Only four of these compounds (phenylthiourea, α-naphthylthiourea, thiouracil and phenylthiocarbamide) have been found to be effective *in vivo*. Phenylthiourea, α-naphthylthiourea or phenylthiocarbamide when administrated to black rats produces depigmentation (90, 91).[7] Removal of these substances from the diet results in a return of pigmentation. Thiouracil given to a patient with generalized melanosarcoma and melanuria has been shown to change the color of the urine from black to normal color (92). In a Negro patient under treatment with thiouracil for hyperthyroidism, areas of depigmentation developed (93).

Competitive Inhibitors

Some derivatives of tyrosine (for example, N-acetyltyrosine, N-formyltyrosine and 3-fluorotyrosine) are effective inhibitors of the tyrosine-tyrosinase and dopa-tyrosinase reactions (57, 58). These substances appear to act as competitive substrates by competing with the natural substrates, tyrosine and dopa, for active centers on tyrosinase. This inhibition has been observed *in vitro* and has not been studied *in vivo*.

[7] When the coat color of animals is considered (for example, in the black rat) the term depigmentation should not be taken to mean that existing melanin pigment in grown fur is decolorized. The term is used only to indicate that new hair does not contain normal amounts of melanin.

Substances That Prolong the Induction Period

Since there is an induction period in the tyrosine-tyrosinase reaction, a delay in the formation of melanin will occur if the induction period is prolonged. In this sense, substances or factors that lengthen the induction period may be considered to be inhibitors of pigment production. The detergent 'Tween-20' prolongs the induction period of the tyrosine-tyrosinase reaction but has no effect on the dopa-tyrosinase reaction (94). If the *p*H of a tyrosinase reaction mixture is increased to more than 7.5 or decreased to less than 6.5 the induction period is markedly increased. No adequate explanation of this phenomenon is known at present. Although to our knowledge, no clear-cut animal experiment or clinical observation has been shown to demonstrate an action of these inhibitors *in vivo* it is possible that examples may be found with -urther investigation.

Substances That Combine with Orthodihydroxy Groups

Sodium molybdate, when fed to cattle, causes a loss of coat color (95, 96). Since molybdate ions are known to combine with *o*-dihydroxy groups, a possible mode of action is by combination of the molybdate ions with compounds such as dopa and interference with their further metabolism (97). This view implies that in the presence of molybdate ions tyrosine can be oxidized to dopa but that dopa cannot be oxidized because it combines with molybdate ions. However, the fact that copper sulfate restores the coat color when given to molybdate-treated cows suggests that inhibition of pigmentation by direct combination of dopa with molybdate may not be an important factor. Molybdate may interfere with the absorption or utilization of copper, or with both.

Substances That Combine with Orthoquinones

In the usual process of melanin formation dopa is oxidized to dopa-quinone and the dopa-quinone is further oxidized. If dopa-quinone is removed from the reaction, melanin production will be inhibited. Aminophenyl compounds such as aniline, 3-amino-tyrosine, and *p*-phenylenediamine combine with orthoquinones and are inhibitors of melanin formation (53, 58). Action of these inhibitors *in vivo* has not been reported.

Reducing Substances

The *o*-quinones can be removed not only by the action of aminophenyl compounds but also by reduction to *o*-dihydroxyphenyl compounds by certain agents. In this way reducing substances can act as inhibitors of melanin pigmentation.

Ascorbic acid is a good example of this type of inhibitor. In the presence of ascorbic acid melanin cannot be formed by the action of tyrosinase on tyrosine or dopa until all the ascorbic acid is oxidized (98, 99). Large doses of ascorbic acid have been reported to decrease the pigmentation in patients with Addison's disease (100, 101, 82). A partial explanation of this phenomenon may be that excess ascorbic acid prevents melanin formation (see pages 106, 119 and 121, 122).[9]

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[9] Ascorbic acid in large doses may also reduce the melanin in the skin to a relatively light-colored substance (82).

Hydroquinones

Compounds such as hydroquinone and *p*-benzylhydroquinone are effective inhibitors of melanin formation both *in vitro* and *in vivo*. Although their mode of action is not clear, they appear to act partly as reducing substances such as ascorbic acid. In addition these substances may act directly on tyrosinase.

Depigmentation in cats, rats and mice (102, 103) has been produced by adding hydroquinone to the diet. This effect of hydroquinone is reversible, since the animals become repigmented when hydroquinone is removed from the diet.

The *p*-benzylhydroquinone ('agerite alba'), which is used as an antioxidant in the processing of rubber, can produce depigmentation of human skin. The events leading to the discovery of this compound as the cause of occupational leukoderma in workers wearing rubber gloves containing agerite alba have been described by Oliver, Schwartz and Warren (104). Hydroquinone is reputed to have a similar but weaker action than does agerite alba. Perhaps the latter chemical is more effective because it is soluble and can penetrate through the skin more readily than can hydroquinone.

NUTRITIONAL FACTORS IN MELANIN FORMATION

It is well established that an abnormal increase or decrease in melanin pigmentation is associated with a variety of nutritional deficiencies. This phenomenon has been observed in several species of animals in addition to man. In some cases of abnormal pigmentation resulting from nutritional deficiency, it is difficult to determine the mechanism of the process. Most of the difficulty arises from the fact that several dietary factors are lacking in deficiency states, and usually it is not possible to relate the pigmentation directly to the lack of a single substance. Only a brief discussion of this interesting subject will be given here. A more detailed report of the literature can be found in a recent review by Frost (105).

Dietary Protein and Amino Acids

In 1923 Hartwell (106) reported that brown-black rats on a diet of bread, whole milk and vegetable kitchen scraps lost much of their color and became grey-fawn or even white. The animals became repigmented after 'food casein' was added to the diet. Hartwell suggested that the depigmentation resulted from a deficiency of tyrosine and tryptophane and showed that this deficiency could be corrected by feeding proteins such as 'food casein' which contain large amounts of these two amino acids. Since melanin is formed from tyrosine, any deficiency of tyrosine should result in a decrease of melanin production. Hartwell's findings can perhaps be partly explained on such a basis. However, the precise compositions of the diets used in her experiments were not reported, and it is not unlikely that a vitamin or mineral deficiency that was cured by 'food casein' also existed.

Rats on a synthetic diet poor in cystine and pantothenic acid have been found to become depigmented (107). Administration of cystine augmented the curative effect of calcium pantothenate. Lysine has been shown to be necessary for normal feather pigmentation in bronze poults (108). No adequate explanation of these findings is available.

It is unfortunate that more work on the amino acid requirements for normal pigmentation has not been carried out, especially with those amino acids that are necessary for the formation of tyrosinase itself.

Vitamins

Rats (108–113), dogs (112, 114), guinea pigs (112) and silver foxes (113) become depigmented when they are given synthetic diets deficient only in the filtrate factors of the vitamin B complex. These factors are not thiamine, riboflavin or pyridoxine and are not adsorbed on Fuller's earth. Pigmentation returns to normal when the animals are given adequate amounts of filtrate factors. In some instances, pantothenic acid, and, to a lesser extent, biotin have curative effects. In other cases pantothenic acid is not effective, but liver extracts containing relatively small amounts of pantothenic acid and yeast are curative. Frost and co-workers (115) suggested that a factor in liver and yeast potentiates the action of pure pantothenic acid. It remained for Wright and Welch (116) to indicate a possible interrelationship between pantothenic acid and pteroylglutamic acid. They showed that hepatic storage of pantothenic acid was increased after administration of folic acid concentrate and biotin to succinylsulfathiazole-fed rats in which depigmentation had developed. From these reports it appears that pantothenic acid is only one, although probably the most important, of the filtrate factors which act synergistically with pteroylglutamic acid in the development of normal pigmentation. Other filtrate factors that appear to play a role in melanogenesis are *p*-aminobenzoic acid and biotin.

In children on a multiple vitamin-deficient diet gray hair and depigmentation of the skin have been found to develop (117). After treatment with injectable liver extracts, powdered stomach and full diets, pigmentation gradually returns.

Definitive statements cannot be made concerning the mechanism by which vitamins of the B complex regulate melanin formation, as discussed previously. Studies *in vitro* of the effect of these factors on the enzymatic oxidation of tyrosine and dopa to melanin may clarify some aspects of this problem.

In contrast to the depigmentation associated with dietary deficiencies of some of the vitamins of the B complex, nicotinamide deficiency (pellagra) often results in increased pigmentation (118). Increased melanin pigmentation in patients with pellagra is seen most commonly at the site of the skin lesions. Pigmentation develops as the acute phase of the dermatitis subsides. The mechanism of this hyperpigmentation appears to be similar to that involved in postinflammatory pigmentation, namely, destruction of sulfhydryl groups during the acute dermatitis with a resulting increase in tyrosinase activity which persists until the concentration of the sulfhydryl group near the melanoblasts returns to normal. This mechanism will be discussed in greater detail in another section.

Increased pigmentation of the skin is also found in patients with sprue (119). The pigmentary signs usually resemble those found in cases of starvation (see later), but at times they may resemble those associated with Addison's disease.

An interesting type of hyperpigmentation is found in patients with vitamin A deficiency (120). The increased pigmentation in these patients is, for the main part, located at the sites of hyperkeratotic follicular papules which are present in this dis-

order. It is possible that the hyperpigmentation results from a decrease in the concentration of sulfhydryl groups in the skin. As will be seen in another section, sulfhydryl groups are *natural* inhibitors of tyrosinase because they combine with copper ions, which are necessary for tyrosinase action. Any reduction in the amount of substances that contain the sulfhydryl group, such as glutathione, near the site of melanin formation (melanoblast) represents a removal of a normal inhibitor of tyrosinase with a resulting increase in melanin production. Since vitamin A deficient patients usually have a diet inadequate in the sulfur-containing amino acids, and since such amino acids are used in keratin formation,[*] it is likely that those amino acids which are available form the protein of the hyperkeratotic papules at the expense of forming glutathione and other sulfhydryl compounds. It would be expected, as a result of this process, that the concentration of glutathione would be decreased in the vicinity of the melanoblast.

Usually little mention is made of pigmentation in cases of vitamin C deficiency, although a few reports (121, 122) indicate that this disorder may be associated with an increase in melanin pigment. In advanced cases of scurvy cutaneous hemorrhages occur, with a resulting increased deposition of hemoglobin breakdown products in the skin. Pigmentation, when it occurs, might result from a decrease in sulfhydryl groups in the skin which would follow an increase in the deposition of iron and copper compounds. This mechanism is similar to that suggested later in explanation of the increased melanin formation of hemochromatosis.

In conclusion, it can be stated that changes in melanin pigmentation are often seen in vitamin deficiency states. Decreased pigmentation is associated with inadequate intakes of the filtrate factors of the vitamin B complex. The mechanism of this process is not known. Increased pigmentation is found in deficiencies of nicotinamide and vitamins A and C and may result from a release of normal sulfhydryl inhibition of tyrosinase. The decrease in sulfhydryl groups can be produced in several ways.

Copper

Evidence from many different types of experimental work shows conclusively that copper is essential for normal pigmentation in mammals. Copper-deficient diets invariably result in depigmentation in rats (124, 125), cats (126), rabbits (126, 127) and cattle (128). Addition of trace amounts of copper salts to the deficient diet restores pigmentation. Other metals (iron, zinc and manganese) and vitamins of the B complex are ineffective by themselves in reversing the depigmentary process, although the administration of copper plus these substances is sometimes more effective than copper alone.

Further evidence for the necessity of copper in animal pigmentation is provided by the interesting reports on chronic molybdenum toxicity in cattle. Muir (95) first described the syndrome of depigmentation, intense diarrhea and emaciation in cattle which grazed on pastures (in Somerset, England) containing excessive amounts of

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[*] It has been suggested (123) that keratin formation represents a normal 'excretory process' for glutathione elimination. According to this view sulfhydryl compounds such as glutathione are thought to be used as a source of cystine, which is necessary for keratin formation. The compounds are eliminated when keratin ceases to be active in metabolic processes.

molybdenum. The disease was cured by the administration of copper sulfate. This syndrome has been experimentally produced in cattle by prolonged feeding of molybdenum. At necropsy a decreased copper content of the liver has been found, indicating that excess molybdate in the diet interferes with copper metabolism.

Since copper is required for tyrosinase activity (60), it is reasonable to assume that a lack of dietary copper results in depigmentation because insufficient copper is available for the normal enzymatic formation of melanin.

Starvation

An unusual pigmentary disturbance of the skin in starvation recently has been described by European authors (129). A splotchy, dirty, grayish brown pigmentation appearing anywhere on the body but most often on the face was seen frequently at the end of World War II. This effect of starvation in humans is of special interest because the intake of several dietary factors such as vitamins, amino acids, fats and minerals is often reduced in a very low caloric diet.

In the latter part of 1944, a severe shortage of food and consequent starvation occurred in Western Holland (130). A dietary survey indicated that the average food consumed per person per day contained about 1000 calories. Since most of the food was obtained from vegetables, it is likely that the diet contained adequate amounts of most vitamins and copper, but inadequate amounts of many amino acids and fats, in addition to the low caloric intake. In these cases the same type of pigmentation developed as described in the preceding paragraph.

The situation in German concentration camps was much more severe (93). The inmates not only had a caloric intake of less than 1000 calories, but also were supplied with inadequate amounts of organic and mineral factors. In these cases a generalized grayish brown pigmentation developed and often there was a melanosis resembling that noted in Addison's disease.

With only the information available at present, it is difficult to account for the increased pigmentation. The fact that much of the increased pigmentation of the group in Western Holland occurred on the exposed areas suggests that ultraviolet radiation may have been a factor. Because of the low dietary intake of sulfur-containing amino acids[10] a decrease in the amount of substances containing sulfhydryl groups, which normally inhibit pigmentation, might be found in the skin. Further speculation does not seem justified until more is known about other factors, especially endocrine. Adrenal insufficiency may have been present.

HORMONAL FACTORS IN MELANIN FORMATION

It is well known that endocrine factors play an important role in melanin pigmentation in man and lower animals. Hence, it is surprising that although many experimental and clinical data have been obtained on this subject from studies on humans and lower species, practically nothing is available from experiments *in vitro*.[11] Nearly all the investigations in this field have been carried out upon intact

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[10] It appears likely that the relative concentration of sulfur-containing amino acids to phenylalanine and tyrosine (which are required for melanin formation) was reduced, because many vegetables have small amounts of cystine and methionine but large amounts of phenylalanine and tyrosine.

[11] This phrase 'experiments *in vitro*' is used here to mean only investigations carried out with isolated enzyme systems. Tissue culture experiments are included with findings *in vivo*.

organisms. In the present discussion efforts will be made to report and correlate information gained from observations on man and lower animals. In addition, the only two reports that we are aware of on studies with isolated enzyme preparations will be carefully evaluated.

Observations on Human Subjects and Experimental Animals

Sex hormones. Estrogens given orally have been shown to induce pigmentation in the nipples and areolae, and along the linea alba in humans (131). Parenteral administration of estrogens to guinea pigs induces pigmentation in the nipples and areolae (131). Particularly interesting is the development of pigmentation of the nipples following local unilateral application of estrogens to guinea pigs (131). This observation suggests that estrogens have a local pigmentogenic action on the melanoblast. The hyperpigmentation observed frequently in the nipples, areolae, linea alba and face during pregnancy has not been satisfactorily explained, but it is believed to be related to the high estrogen levels during gestation. The lack of pigmentation following administration of large doses of estrogens to women in the menopause is considered by Davis and co-workers (131) to be due to anatomic and functional changes in the pituitary occurring in the processes of aging, which interfere with the development of hyperpigmentation.

Testosterone induces melanin pigmentation when applied locally to sparrows' bills (132) and to the scrotum of the ground squirrel (133). Injection of androgens also increases melanin pigmentation in human male castrates (134). It has been observed that in male castrates little increased pigmentation develops upon exposure to ultraviolet light (134). However, if these men are given testosterone propionate several days after exposure to ultraviolet radiation, hyperpigmentation develops over the exposed areas.

Forbes (135) has reported darkening of the hair in male and female rats after implantation of both androgenic and estrogenic hormones in the form of pellets. Hamilton (136) working with tissue cultures of skin ectoderm from fowls found that androgens and estrogens accelerated the differentiation of melanophores.

As mentioned previously, estrogens and androgens appear to increase melanin pigmentation by acting directly on the melanoblasts. Their exact mode of action is obscure, and speculation on this subject is not justified at the present time.

Pituitary hormones. Cold-blooded vertebrates show striking pigmentary responses to changes in illumination, temperature and other factors (137). These alterations in pigmentation result from the expansion and contraction of dermal and epidermal melanophores, regulated by nervous and humoral influences. The humoral control is due to a blood-circulated pituitary melanophore hormone (or hormones). Injection of melanophore hormones into hypophysectomized frogs causes expansion of the cutaneous melanophores. Pituitary extracts from vertebrates of several classes, including mammals, contain melanophore hormones. It is interesting that although mammals do not have cutaneous melanophores, a rich store of melanophore-expanding hormone (intermedin) exists in the pituitary gland. Dawes (138, 139) has provided evidence that when amphibians with active melanophores are maintained for prolonged periods on illuminated black backgrounds, an absolute increase in amount or darkening in color of melanin results.

Clinical evidence indicates that the pituitary gland has some control of pigmentation in man. Patients with hypopituitarism often exhibit decreased melanin pigmentation. It is not known whether the pituitary gland exerts a direct or an indirect effect on pigmentation.

Adrenal hormones. Hyperpigmentation in animals following adrenalectomy has been observed by Ralli and Graeff (140, 141) and by Butcher (142). The former investigators produced nutritional achromotrichia in rats on a diet deficient in the filtrate factor and noted that adrenalectomy resulted in repigmentation of the hair. This repigmentation could be prevented by giving the animals desoxycorticosterone. In humans, also, total removal of the adrenal cortical tissue by surgery produces the clinical picture of Addison's disease with deep hyperpigmentation (143). These facts suggest that adrenal hormones under certain conditions have an inhibitory effect on melanin pigmentation. Hamilton (136) has demonstrated an inhibitory action of desoxycorticosterone on the development of melanophores in explants of skin from chick embryos grown in tissue culture. A recent report by Whitaker and Baker (144) showed the inhibitory effect of locally applied 11-dehydro-17-hydroxycorticosterone on melanin pigmentation and hair growth in black-hooded rats. In spite of these experimental findings, the exact mechanism of increased melanin pigmentation in adrenal hypofunction (Addison's disease) is not yet satisfactorily explained. The hyperpigmentation in this syndrome only rarely decreases and most often remains unchanged after replacement therapy with either adrenal cortical extracts or synthetic hormones such as desoxycorticosterone or 11-dehydro-17-hydroxycorticosterone. It is possible that the adrenal fraction responsible for the inhibitory effect of the adrenal gland on pigmentation either had been destroyed in preparation of the extracts or was present in insufficient quantity.

The slowly developing diffuse hyperpigmentation frequently associated with Addison's disease usually affects the parts of the body that are normally hyperpigmented, such as the axillae, areolae and anogenital regions. Exposed parts of the body such as the face and parts subjected to mechanical irritation also show increased pigmentation. The hair frequently darkens. Pigment normally present in the oral mucosa is increased in many patients. It is interesting that, although patients with Addison's disease have a generalized tendency toward hyperpigmentation, this abnormally increased deposition of pigment occurs in regions of the body most favored for normal melanin formation.

Areas of skin and mucous membrane, such as the axilla, groin, skin folds and oral cavity, which are at higher temperature than other regions, tend to be hyperpigmented. This might be associated with the fact that the rate of enzymatic oxidation of tyrosine to melanin like most enzyme reactions is dependent on temperature. The reaction is much more rapid at temperatures greater than 30° C. This subject will be discussed in more detail later in the paper.

Other hyperpigmented parts, such as the face and the dorsum of the hands, are exposed to light, which is known to stimulate melanin formation.

Much speculation has arisen concerning the mechanism of the processes which predispose to hyperpigmentation in patients with Addison's disease. According to

one of the oldest, and discarded, hypotheses, it is assumed that inhibition of the sympathetic nervous system results in increased melanin formation and that normally the adrenal gland stimulates the sympathetic system (145). Adrenal insufficiency would then predispose one to increased pigmentation due to lack of adrenal stimulation.

Bloch and Löffler (146) supported the view that epinephrine and melanin are derived from the same precursor and that if the adrenal glands do not utilize this substance for synthesis of epinephrine, it accumulates and is converted to melanin with subsequent melanosis.

Another hypothesis was proposed when it was found that ascorbic acid, normally present in high concentration in the adrenal gland, is decreased in Addison's disease presumably because of the destruction of adrenal tissue. Ascorbic acid is assumed to be a normal inhibitor of melanin formation, and any decrease in ascorbic acid in the tissues would result in hyperpigmentation (147). There is good evidence that this hypothesis is not correct, for if ascorbic acid normally inhibits melanin formation, dopa should accumulate in the serum. This has not been found to be the case (99).

Calkins (94) has suggested that the pituitary may have a primary role in human melanogenesis and that there may exist a pituitary-adrenal interrelationship (similar to the pituitary-thyroid axis) whereby adrenal hormones inhibit the release of intermedin (pituitary melanophore hormone). Primary adrenal cortical insufficiency would result in a compensatory overactivity of the pituitary with an increased output of intermedin followed by increased melanin synthesis. This hypothesis is interesting in view of some recent evidence (148) demonstrating an increased adrenocorticotrophic activity in the blood of patients with adrenal cortical insufficiency (Addison's disease). It is a well-known clinical observation that patients with secondary adrenal cortical insufficiency as a result of hypopituitarism (Simmonds' disease) only rarely develop hyperpigmentation.

An interesting observation,[12] which may indicate a possible role of the pituitary in human melanogenesis, has been made in a patient receiving adrenocorticotrophin (ACTH) in the arthritic service of the Mayo Clinic. This patient, a white male, developed marked pigmentation of the palmar creases, axillae, dorsum of the hands and feet, and in a recent operative scar. There were no indications of adrenal insufficiency which might account for the development of the pigmentation. The ACTH preparation, however, was found to contain appreciable melanophore hormone (intermedin) on bioassay in hypophysectomized frogs, a finding which may be relevant to the development of pigmentation under chronic treatment with pituitary preparations.

The authors would like to suggest that in Addison's disease there may be a decrease in concentration of sulfhydryl groups at the site of melanin formation. A decrease in sulfhydryl compounds in the blood of patients with Addison's disease has been reported (149). This observation indicates that the adrenal glands may

play a role in regulating the metabolism of sulfhydryl compounds. As stated previously a decrease in glutathione or similar substances in the skin would tend to increase melanin pigmentation.

Several variations of the foregoing hypotheses can be found. It remains to be seen which if any of these views will receive experimental support.

Enzyme Studies

In 1940 Fostvedt (150) reported results of detailed experiments concerning the influence of melanophore hormones on the tyrosine-tyrosinase reaction. He worked with mealworm tyrosinase and melanophore hormone preparations obtained from the pituitary glands of different species of land and marine animals. Some hormone preparations apparently accelerated the tyrosine-tyrosinase reaction, as determined by oxygen uptake measurements, whereas others inhibited it. None of the preparations, however, had a notably marked effect on the tyrosine-tyrosinase reaction.

Fostvedt's systems had only weak tyrosinase activity, and usually no effect was noticeable for one or two hours. He reported differences between the experimental and control studies after this time, experiments sometimes being carried out for six hours. However, throughout the experiment the slopes of the oxidation curves for the test and control runs did not differ greatly, and it is difficult to ascertain whether an accelerating or inhibiting effect was present. Experiments should be done in which the tyrosinase activity is varied and the hormone concentration is kept constant, and vice versa. Also, since Fostvedt found relatively little leveling off of the oxidation curves even after 60 per cent of the tyrosine was oxidized, it would be interesting to prolong the experiments to determine whether or not all the oxygen consumed is used only to oxidize tyrosine to melanin. Fostvedt's experiments represent an interesting beginning in this field, but more work must be done before definite conclusions can be reached.

Figge and Allen (151) reported in 1941 that the inhibition of melanin formation by glutathione was released by estrone. Working with crude potato tyrosinase preparations, they measured melanin production in the tyrosine-tyrosinase reaction by colorimetric means. Glutathione prevented the formation of melanin, and estrone (in equimolar quantities with respect to glutathione) reversed the inhibition. This finding, if correct, would be of immense significance and might account for some of the action of estrone on pigmentation *in vivo*. Unfortunately, Figge and Allen did not use adequate controls since they did not determine the action of their tyrosinase preparations on estrone itself. Additional experiments, including some with purified tyrosinase, must be carried out to determine the effect of estrone on the tyrosine-tyrosinase reaction. The crude enzyme preparation probably contained estrinase (152). It would be interesting to know whether an oxidation product of estrone released the glutathione inhibition of tyrosinase or whether estrone itself was oxidized to a colored product.

NEUROGENIC FACTORS IN MELANIN FORMATION

In addition to the influence of nutritional and hormonal factors on the biochemical reactions involved in melanin formation, certain neurogenic factors play im-

portant roles. Most of the information on the neurogenic controls of pigmentation has been obtained from clinical observations.

A neurogenic control of melanogenesis in human skin is suggested by some recent transplantation experiments reported by Haxthausen (152a). When normal skin was transplanted to an area of vitiligo, the graft was found in a few months to become depigmented. Conversely, when the vitiliginous skin was transplanted to an area of normal skin, the pathologic skin graft gradually repigmented.

The hyperpigmentation seen in patients with the rare disease, acanthosis nigricans, is similar to that observed in Addison's disease and often develops when a malignant lesion of the viscera, either by direct extension or through pressure, involves the celiac plexus or chromaffin system. Reports have been made of abnormal pigmentation associated with neurologic and psychiatric disturbances (153–155).

These and other findings indicate that abnormalities of the nervous system can result in disturbances in pigmentation. The mechanism of the neurogenic control is unknown. It is hoped that some aspects of this fascinating subject can be clarified in the near future.

BIOCHEMICAL BASIS FOR MELANIN FORMATION IN CLINICAL CONDITIONS

The elaboration of melanin pigment in the epidermal melanoblast ordinarily depends on the available concentration of three substances: *1*) the enzyme tyrosinase —a copper-protein complex attached to ultramicroscopic particles in the cytoplasm of the melanoblast; *2*) a suitable substrate—usually tyrosine or dopa; *3*) molecular oxygen. If any of these substances is absent, the formation of melanin is impaired. This conclusion is self-evident when one considers the reactions involved in the oxidation of tyrosine to melanin as shown in figure 1.

The following are some disorders that deviate from this rule because of additional factors. As discussed earlier, dihydroxyphenyl compounds such as dopa or epinephrine can be fairly rapidly oxidized to deeply pigmented substances by molecular oxygen under certain physiologic conditions even in the absence of tyrosinase. In alkaptonuria, a paradihydroxyphenyl compound, homogentisic acid, is present in large amounts in body tissues and fluids (156). The oxidation of this substance in urine exposed to air (and possibly also in body tissues) produces a black pigment. A second example is the spontaneous oxidation of 5,6-dihydroxyindole to melanin in the tissues of patients with metastatic melanoma and melanuria. This will be discussed later.

It has been reported that dopa can be oxidized to melanin *in vitro* in the presence of an active cytochrome C-cytochrome oxidase system in the absence of tyrosinase (157). There is no indication that such a system plays any role in normal melanin formation *in vivo*. The dopa-tyrosinase reaction is extremely rapid, and since melanin is usually produced only in certain specific cells which contain tyrosinase, it is unlikely that a cytochrome system plays a direct role in melanin formation. When melanin is produced in tissues which do not themselves contain melanoblasts, as in cases of alkaptonuria with ochronosis and some cases of generalized melanosis, the possibility exists that the cytochrome systems play a more direct part. But even in these cases there is no need to postulate that the reactions would depend completely on these systems.

The reaction of the three basic substances, tyrosinase, substrate and molecular oxygen, is controlled by several physicochemical factors which determine the rate of melanin formation: *1*) a catalytic substance, usually dopa, which can accelerate the tyrosine-tyrosinase reaction; *2*) chemical groups which normally inhibit copper en-

zymes, for example, sulfhydryl groups, normally found in the epidermis; *3*) physical and chemical factors such as temperature, hydrogen ion concentration and oxidation-reduction potentials. The quantity of melanin produced by the cell depends on the over-all balance of these different forces as illustrated in figure 3.

Many substances, including dihydroxyphenyl compounds and reducing agents, catalyze the tyrosine-tyrosinase reaction. Of these substances dopa is the most effective now known (57, 58). The mechanism of this catalytic process was discussed previously.

It was pointed out in an earlier section that several compounds inhibit tyrosinase. Some effective inhibitors are naturally occurring organic sulfur compounds, such as glutathione and cysteine. These substances inhibit the enzyme by binding copper which is necessary for tyrosinase action. Ginsburg (158) demonstrated the presence

BIOCHEMICAL FACTORS REGULATING THE FORMATION OF MELANIN
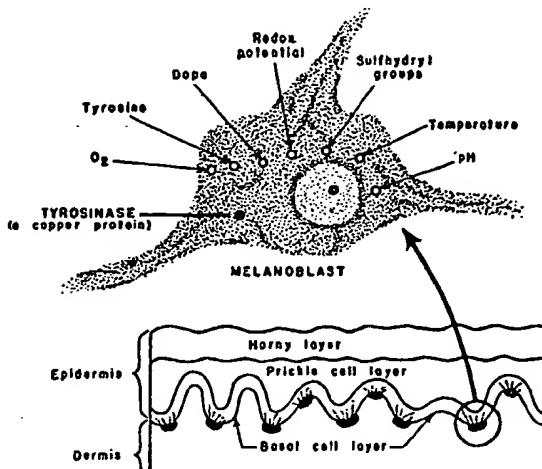
Fig. 3. Factors regulating the formation of melanin.

of sulfhydryl compounds in extracts of guinea pig skin, and recently Rothman and co-workers (29, 30) have found similar substances in isolated human epidermis. These findings suggest that sulfhydryl compounds occurring naturally in the skin may retain the enzyme tyrosinase in an inactive state by binding copper. Oxidation or inactivation of the sulfhydryl groups releases the bound copper, thus facilitating tyrosinase action.

From data already presented it can be seen that increasing the temperature, up to certain limits, of a tyrosine-tyrosinase reaction accelerates the reaction. The hydrogen ion concentration is also an important factor in melanin formation. The pH range 6.7 to 7.2 appears to be optimal. At higher values of pH the induction period in tyrosine oxidation is prolonged, and at lower values of pH tyrosinase activity is reduced. An additional factor is the oxidation-reduction potential of the system, which

may control the tyrosine induction period. High redox potentials are associated with long induction periods.

The state of oxidation of melanin itself may be important in the pigmentation of the skin. Melanin is light colored in the reduced form and black in the oxidized form. In the presence of reducing substances such as ascorbic acid, melanin is light colored (see page 106).

Most investigations of the enzymatic formation of melanin have been carried out with plant, insect and mammalian melanoma tyrosinase. Relatively little work has been done with tyrosinase from human epidermis (see pages 104, 105). For this reason only the factors which appear to be applicable to tyrosinase from all sources are discussed. It is assumed that the data apply to human epidermal tyrosinase as well. Justification for this assumption arises not only from the observation that some properties are common to all tyrosinases but also from the fact that tumors have not been found to contain enzymes differing from those present in normal tissues. There is no reason to believe that melanoma tyrosinase, which can easily be prepared, differs from normal skin tyrosinase, which can be isolated only in small quantities.

On the basis of the foregoing data attempts will be made to explain some normal and abnormal changes in pigmentation other than changes associated with nutritional, hormonal and neurogenic disturbances. It is not the purpose of this paper to review all known pigmentary disorders. Only conditions which illustrate biochemical regulatory factors will be discussed.

Suntanning

Ultraviolet irradiation appears to be concerned with melanin formation in at least four different ways, all of which tend to increase pigmentation. First, ultraviolet radiant energy catalyzes the oxidation of tyrosine to dopa (159). Small amounts of dopa thus formed can then catalyze the tyrosine-tyrosinase reaction. Second, the concentration of sulfhydryl groups in human epidermis is decreased after irradiation with ultraviolet light (160). Ionizing radiations (ultraviolet, roentgen, radium) produce an oxidizing agent (or agents) from water, which can oxidize sulfhydryl groups. In this process natural inhibitors of tyrosinase are removed. Third, the redox potential of human skin (161) decreases appreciably after irradiation. As stated previously, tyrosinase may be more active at relatively low potentials than at high ones. Fourth, cutaneous temperature is often but not always increased when one is exposed to ultraviolet irradiation, as in direct exposure to the sun on a warm, clear day. In addition, the erythema of the skin which results from exposure to ultraviolet light may elevate epidermal temperatures to values above normal. As already mentioned, increase in temperature accelerates melanin formation. The interrelationship of the various factors concerned in melanin formation by ultraviolet radiation are summarized in figure 4. In addition to its four probable effects on melanin formation, ultraviolet radiant energy also causes darkening (by oxidation) of melanin already present in the skin. A more detailed discussion of this interesting topic has recently been reported (162).

Heavy Metals and Melanosis

Increased melanin pigmentation is frequently observed when heavy metals such as arsenic, bismuth, iron, gold, silver[13] and mercury are deposited in the skin (163). Deposition of metals in the skin usually occurs when drugs containing heavy metals are used therapeutically. Patients with hemochromatosis have relatively large amounts of iron and copper deposited in the skin (164). The most reasonable explanation for these findings is that metals bind epidermal sulfhydryl groups and thereby release inhibition of tyrosinase. The increased tyrosinase activity results in increased melanin formation.

MELANIN FORMATION BY ULTRAVIOLET RADIATION
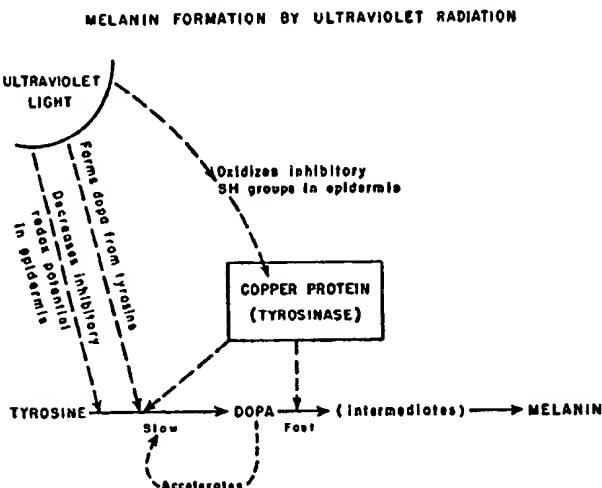
Fig. 4. FACTORS controlling the formation of melanin by ultraviolet radiant energy.

Heat and Pigmentation

Localized pigmentation often follows burns or chronic exposure to heat of limited areas of skin. The increased temperature may accelerate melanin pigmentation directly by accelerating the enzymatic oxidation of tyrosine and by increasing the rate of sulfhydryl group oxidation. After severe burns with destruction of melanoblasts, decreased pigmentation is noted. In these cases the enzyme tyrosinase is absent and melanin cannot be formed.

Postinflammatory Pigmentation

Increased pigmentation is seen in many patients who have had cutaneous inflammatory diseases. Rothman and co-workers (29) suggested that sulfhydryl compounds are oxidized or otherwise destroyed in some of the inflammatory processes with the result that melanin formation is increased.

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[13] The presence of silver in the skin (argyria) may be associated with only a slight increase in melanin pigmentation. Instead, there is often increased pigmentation due to the actual deposition of metallic silver. Small amounts of metallic silver may dissolve and be converted to silver ions. These ions could react with sulfhydryl groups to produce the slight increase in melanin.

Hyperthyroidism

Increased pigmentation has been noted in some patients with hyperthyroidism and decreased pigmentation in others (165). It is difficult at this time to interpret these conflicting observations. Hyperpigmentation might be related to the decrease in sulfhydryl compounds which has been reported in hyperthyroidism. Hypopigmentation might be due to decreased tyrosine concentration in the skin as a result of increased conversion of tyrosine to thyroxine and other substances due to the hypermetabolic state. The balance between the relative concentration of sulfhydryl compounds and tyrosine may determine the pigmentary changes in patients with hyperthyroidism.

Melanuria and Generalized Melanosis

It is not unusual to observe melanuria and rarely generalized melanosis in patients with metastatic melanoma (166-174). Linnell and Raper (175) showed that a simple derivative of 5,6-dihydroxyindole was present in the urine of a patient with a metastatic melanoma and melanuria. Melanotic tumors may produce such large amounts of the oxidation products of tyrosine that some of these compounds (dopa, 5,6-dihydroxyindole, etc.) are released into the general circulation before being completely oxidized to melanin in the tumors. These substances could be oxidized to melanin in tissues distant from the tumor site even in the absence of tyrosinase (fig. 1).

Phenylpyruvic Oligophrenia

Phenylpyruvic oligophrenia is a rare disease in children characterized by mental deficiency and increased urinary excretion of phenylpyruvic acid and phenylalanine (176). These patients characteristically have light skin, blond hair and blue eyes (177-179). They do not tan when exposed to sunlight. This condition is considered to be an inborn error of metabolism in which phenylalanine cannot be converted to tyrosine. It is possible that tyrosine, the substrate in the enzymatic formation of melanin, is present in amounts inadequate for melanin synthesis.

Normally, metabolic requirements for tyrosine are fulfilled directly from dietary sources and from oxidation of phenylalanine. Patients with phenylpyruvic oligophrenia obtain tyrosine only from the diet, and they have reduced amounts of tyrosine in the blood. It is possible that the available tyrosine is utilized for the production of essential hormones, (for example, thyroxine) and proteins rather than for conversion to melanin.

Albinism

Partial or complete absence of melanin has been found to occur as a recessive trait in all mammals that have been studied. The inability of albinos to form melanin results from absence of melanoblasts in the epidermis. Since tyrosinase is contained within the melanoblast, the substrate tyrosine, although present in adequate amounts, is not catalytically oxidized to melanin.

Normal Variations in Pigmentation

A question often asked is why certain individuals are more pigmented than others. In normal individuals, pigmentation varies from very light to near black. Among

the factors controlling melanin formation, shown in figure 3, the tyrosine, dopa and oxygen concentrations and the temperature and p_H would be expected to be approximately the same for all healthy people. The concentrations of tyrosinase and sulfhydryl compounds (and possibly the redox potential) are likely to vary in different individuals and to depend in large part on hereditary factors. The state of oxidation of melanin itself may vary. It is not possible at this time to conclude which of these factors plays the dominant role in determining the pigmentation of an individual.

SUMMARY

In this review an endeavor has been made to describe the biochemical mechanisms of melanin formation and to correlate the enzymatic processes involved therein with experimental and clinical observations on melanin pigmentation.

The enzyme tyrosinase, a copper-protein complex, catalyzes the oxidation of tyrosine to dopa and the oxidation of dopa to melanin. Tyrosinase is widely distributed in nature and can be found in plant, insect, marine animal and mammalian tissues. In all these species tyrosinase plays an important role in melanin pigmentation.

Until recently it was believed that mammalian tissue did not contain tyrosinase but contained instead, an enzyme called 'dopa-oxidase,' which supposedly catalyzed only the oxidation of dopa to melanin. It is now known that the original distinction between tyrosinase and dopa-oxidase is no longer valid. Hence, it is suggested that the single term tyrosinase should be used instead of the separate terms tyrosinase and dopa-oxidase.

While tyrosinase obtained from different species has some unique properties depending on the particular source, three characteristics are common to tyrosinase, under proper conditions, regardless of its origin: *1*) the oxidation of tyrosine to melanin is catalyzed by tyrosinase; *2*) the tyrosine-tyrosinase reaction is catalyzed by some *o*-dihydroxyphenyl compound, for example, dopa; *3*) the activity of the enzyme is associated with copper ions. Current investigations indicate that tyrosinase is a single enzyme, although unequivocal proof of this is lacking. The preparation and properties of tyrosinase are discussed.

Various substances inhibit melanin formation *in vitro* and *in vivo*. The mechanism of the inhibition depends on the particular step which is blocked of the tyrosinase-catalyzed series of reactions by which tyrosine is converted to dopa, and eventually to melanin. Melanin pigmentation in mammals is regulated by biochemical factors, some of which are well defined, such as the concentrations of enzyme, substrate, hydrogen ions, sulfhydryl groups, and so forth. Some of these factors, as well as others which are as yet unknown, are influenced by nutritional, hormonal and neurogenic control. The biochemical basis for melanin pigmentation in several clinical conditions has been presented.

Earle Arnow, E. Calkins, A. C. Curtis, R. Dorfman, W. K. Jordan, E. C. Kendall, P. Kruhoffer, A. Lazarow, J. Meyer, H. Montgomery, P. A. O'Leary, S. Rothman, R. G. Sprague, W. H. Summerson, A. D. Welch and H. G. Wood.

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Expression of the Pancreatic Elastase I Gene in Transgenic Mice

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The exocrine pancreas synthesizes, stores, then secretes massive amounts of hydrolytic enzymes for digestion. The synthesis of the secretory enzymes accounts for approximately 80% of the protein synthesis of the entire gland (1, 2). These secretory enzymes, with few exceptions, are not expressed at significant levels in nonpancreatic tissues; the occurrence of similar enzymic activities in other tissues is generally due to the presence of enzymes encoded by different genes. It is the high and selective expression of the genes for a few hydrolytic enzymes that largely determines the differentiative phenotype of the acinar cell, the predominant cell type of the pancreas. To approach an understanding of tissue-specific gene regulation, we have chosen to study the expression of a family of pancreas-specific genes—the pancreatic serine proteases—because of their selective expression and the advantages that accrue from the comparative analysis of a group of similarly regulated genes.

The pancreatic subfamily of serine proteases comprises nine structurally and functionally homologous enzymes (three chymotrypsins, three trypsins, two elastases and one kallikrein). The gene family that encodes these proteins has evolved from a common ancestral gene through a series of duplications (3). With the exception of kallikrein (4), the presence of similar protease activities in nonpancreatic tissues appears due to products of related but distinct serine protease genes. Moreover, expression in pancreatic acinar cells is very high, such that about 20% of the total protein synthesis is for these few serine proteases (2).

To understand the molecular aspects of cell differentiation, it is necessary to define the mechanisms that determine the timing, the extent and the tissue-specific nature of transcription of developmentally regulated genes. An understanding of transcriptional regulation will include knowledge of the nature of DNA control sequences, the number and nature of trans-acting factors that interact with those sequences, the nature of that interaction and how that interaction then modulates gene transcription. Our approach has been to characterize pancreas-specific genes directly and to analyze their mode of regulation through the reintroduction of the cloned, purified genes into animals.

mRNAs OF THE EXOCRINE PANCREAS

As would be expected for a tissue that synthesizes a few prominent protein products, the polyadenylated mRNA population of the pancreas is dominated by a few mRNAs. Figure 1 shows the profile of polyadenylated mRNA isolated from total pancreatic RNA and resolved by electrophoresis. A small amount of 28S and 18S ribosomal RNA remains in this mRNA preparation. The other bands represent the mRNAs for the major exocrine secretory enzymes. The mRNAs for the major secretory enzymes have been identified by two approaches. In many instances, the mRNAs were separated either by electrophoresis (5, 6) or sucrose gradient centrifugation (7) and translated in vitro. The translation products were subsequently identified by immunological techniques or by their characteristic electrophoretic mobilities. Alternatively, cDNA clones, once identified for individual mRNAs, were used as hybridization probes in RNA blot analyses of pancreatic mRNA resolved by electrophoresis (8–12). The mRNAs for elastase II, three chymotrypsins, three trypsins and kallikrein comprise a group of prominent RNAs ranging in length from 900 to 1,100 nucleotides that are not resolved by electrophoresis (Figure 1). Elastase I mRNA is slightly longer (1,250 nucleotides) and is separated from the other mRNAs.

The mRNAs for six pancreatic serine proteases have been cloned as double-stranded complementary DNAs (ds-cDNAs). The cloned ds-cDNAs were identified initially by hybridization on Northern blots to prominent mRNAs of the appropriate size (about 1,000 nucleotides in length). Their identities were confirmed by comparison of the amino acid sequences derived from the nucleotide sequences of the ds-cDNAs with the known amino acid sequences of serine proteases of cow and porcine pancreas.

The serine protease mRNAs share several characteristics (Figure 2). Each mRNA encodes a pre-pro-enzyme. The amino-terminal pre-, or signal, peptide is a characteristic of most secretory proteins and specifies the vectoral transfer of the newly synthesized protein into the cisternae of the rough endoplasmic reticulum as the first step in secretion. The pro-, or activation, peptide ensures the synthesis of an inactive zymogen, and must be

Pancreatic mRNAs
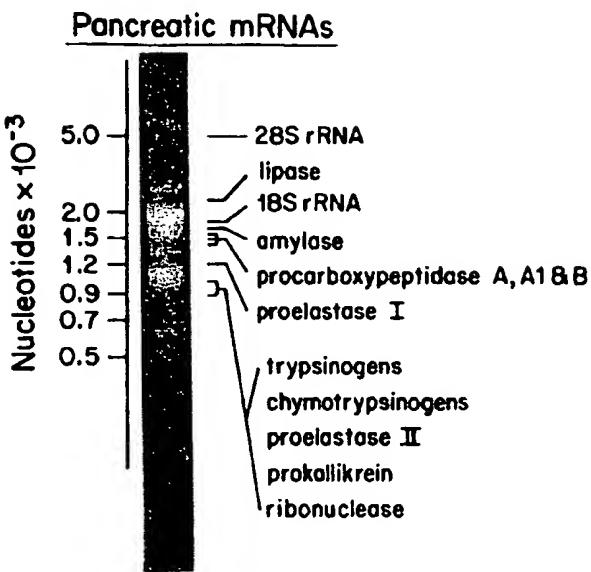
FIG. 1. Prominent mRNAs of the secretory enzymes of the rat pancreas. Total RNA was isolated from rat pancreas by the guanidine thiocyanate procedure of Chirgwin et al. (6). Polyadenylated RNA was selected by affinity chromatography on oligo-(dT) cellulose (30), and resolved by electrophoresis in a 1.5% agarose gel containing the denaturant methyl mercury hydroxide (31). The gel was stained with ethidium bromide and photographed.

cleaved after secretion to yield the active, mature enzyme. In addition to the pre-pro-enzyme coding domain, each serine protease mRNA contains short 5'- and 3'-untranslated regions. The 3'-untranslated region of the elastase I mRNA is relatively longer (280 nucleotides) and accounts for the greater overall length of elastase I mRNA compared to the other serine protease mRNAs.

TISSUE-SPECIFIC EXPRESSION OF THE PANCREATIC SERINE PROTEASES

A characteristic of the pancreatic serine protease gene family is the very high level of expression in the pancreas in contrast to the near absence of expression in other tissues. This differential expression is a consequence of the need to produce massive amounts of the enzymes for digestion in the intestine, and to prevent the expression of even small amounts of these potent and dangerous hydrolytic enzymes in inappropriate tissues. This rigorous, tissue-specific control of a gene family provides an opportunity to study very tight regulatory mechanisms.

A measure of the tissue-specific expression of a representative member of the serine protease gene family (elastase I) is given in Table 1. In the pancreas, elastase I mRNA comprises about 1% of the total mRNA, or about 10,000 mRNAs per average pancreatic cell. Levels are at least 1,000-fold lower in some tissues such as intestine, kidney and liver. The physiological significance of the low but detectable expression in these tissues is unclear, but may represent expression at high levels in a small number of specialized cells, such as tissue mast cells (10). In other tissues such as the parotid and submaxillary gland, elastase I mRNA is undetectable;

expression is at least 100,000-fold lower in these tissues compared to the pancreas. The elastase I gene appears maximally on in the pancreas and is off in tissues such as the parotid and submaxillary glands.

At least one level of regulation of the elastase I gene is transcriptional. Nuclear run-on experiments detect elastase I gene transcription in isolated pancreatic nuclei but not liver nuclei (Figure 3). Elastase I gene transcription in isolated pancreatic nuclei accounts for about 0.2% of the total, whereas transcription of a liver-specific transcript, albumin, is undetectable. Conversely, elastase I gene transcription is undetectable in liver nuclei in which albumin transcription is prevalent. The limitations of the in vitro run-on transcription assay prevent confident measurements of transcription below 1 ppm, so that the maximum level of differential transcription appears to be only 150-fold, but is probably much greater. Therefore, differential rates of transcription alone may account for the disparate elastase I mRNA levels of the pancreas and liver.
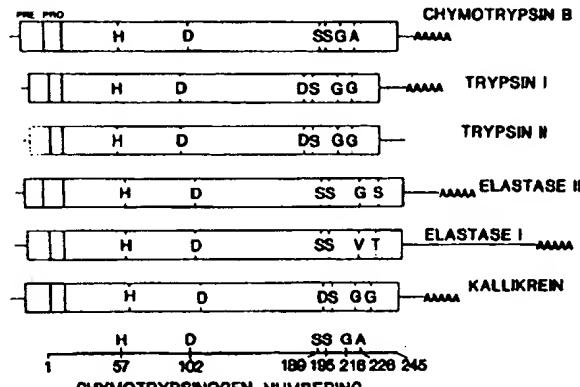
FIG. 2. The structure of pancreatic serine protease mRNAs. Complete mRNA sequences were derived from cloned ds-cDNAs of the enzymes shown. The rectangles delimit the amino acid coding domains of the pre-pro-enzymes; the capital letters indicate the positions of the amino acid residues of the catalytic triad and the primary determinants of substrate cleavage specificity characteristic of each protease subclass. The horizontal lines extending from the rectangles indicate the length of 5'- and 3'-untranslated regions. The dotted lines in the trypsin II mRNA represent regions not yet sequenced. The mRNA cloning and sequencing information were derived from Swift et al. (11) for kallikrein, MacDonald et al. (10) for elastases I and II, MacDonald et al. (8) for trypsins I and II, and Bell et al. (12) and S. Lowther (unpublished data) for chymotrypsin B.

TABLE 1. Elastase I mRNA levels in the rat

	mRNAs per cell
Pancreas	10,000
Liver	10
Kidney	4
Intestine	3
Spleen	0.1
Submaxillary gland	<0.5
Parotid	<0.1
Testes	<0.1

mRNA levels were quantified by solution hybridization using a single-stranded rat elastase I cDNA probe. The numbers of elastase I mRNA molecules per cell were calculated as described by Swift et al. (20).
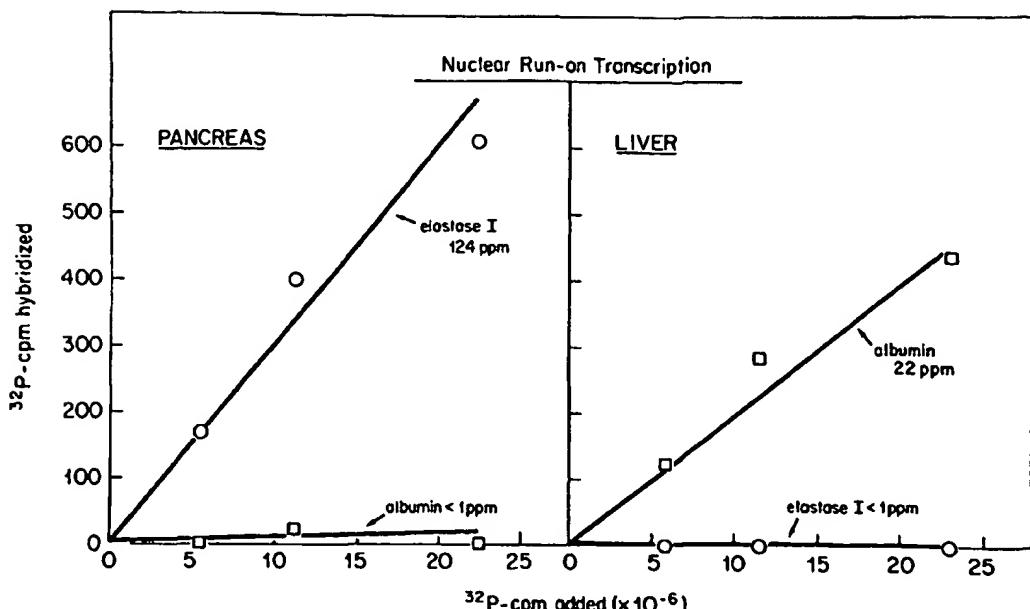
FIG. 3. Pancreas-specific transcription of the elastase I gene. Nuclei isolated from rat pancreas and liver were incubated with [³²P]UTP to extend nascent RNA transcripts. The *in vitro* transcribed RNA was isolated and hybridized to rat elastase I and albumin cDNA plasmids bound to nitrocellulose. Background was defined as the amount of hybridization to the cloning vector pBR322. The relative transcription rates (ppm) are expressed as the amount of hybridizing cpm minus background divided by the number of input cpm and the length of the cloned cDNA probe in kilobases. All procedures were performed as described by McKnight and Palmiter (32).
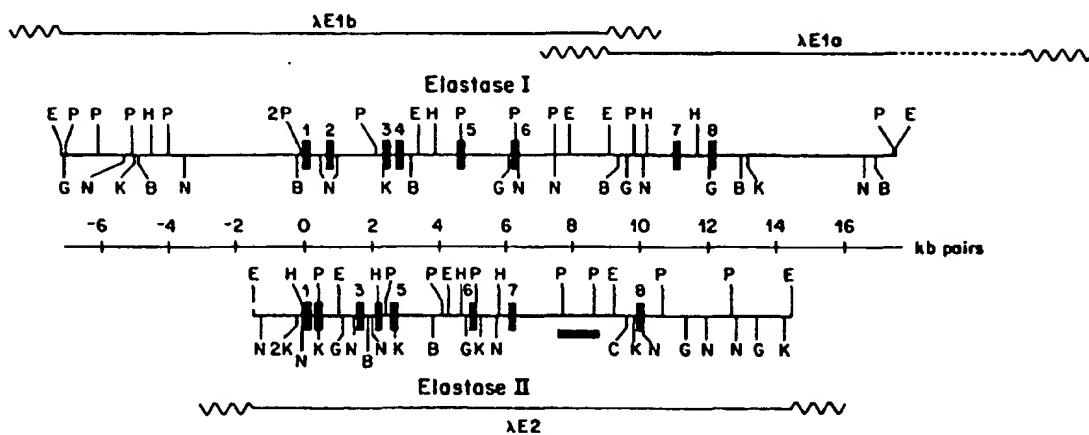
FIG. 4. The sequence organization of the rat elastase genes. The positions of exons shown relative to a restriction map of each gene (E, *Eco*RI; G, *Bgl*II; P, *Pvu*II; N, *Nco*I; K, *Kpn*I; B, *Bam*HI; H, *Hind*III). Elastase clones E1a, E1b and E2 were identified and isolated from a recombinant λ-bacteriophage library (15); the extent of rat genomic sequences is indicated by the *straight lines* and the λ vector by the *wavy lines*.

SEQUENCE ORGANIZATION OF THE SERINE PROTEASE GENES

As for most nuclear genes of higher eukaryotes, the genes encoding the pancreatic serine proteases are split. Trypsin (13) and kallikrein (14) genes are divided into five exons, the chymotrypsin gene (12) into six. The elastase I and II genes are more complex (15). Both are divided into eight exons that extend the length of the genes about 10-fold over the lengths of the mRNAs (Figure 4). The variation in the number of introns among a family of genes that clearly arose from a common ancestral gene indicates that introns have been acquired or lost (or a combination of both) during the divergent evolution of these genes. The entire elastase I gene (Figure 4) was cloned in two recombinant λ-phage which contained genomic sequences that abut in the rat genome (15). In addition to the 12 kb of exon and intron sequences, the cloned gene region contains 7.2 kb of 5'-flanking and 5 kb of 3'-flanking sequences. We chose to characterize further the expression of the elastase I gene because the presence of these extensive flanking regions

increased the likelihood of retaining pancreas-specific regulatory elements.

INTRODUCTION OF THE CLONED RAT ELASTASE I GENE INTO MICE

Two methods are currently available to test for cell-specific expression of cloned genes. One approach is to introduce a purified gene into cells in culture by trans-fection. Cell-specific expression is measured by the differential expression of a gene in an appropriately differentiated cell line (e.g., the chymotrypsin gene in a pancreatic acinar cell carcinoma line) compared to an inappropriate cell line (e.g., a fibroblast or kidney cell line) (16). Major advantages of this method are the relative ease with which multiple genes or altered gene constructs can be tested and the speed of the assay. However, it is impractical to screen a large number of differentiated cell lines representing many different cell types to measure the extended specificity of expression in a broad range of cell types. Moreover, cells in culture tend to express all transfected eukaryotic genes regardless of cell specificity at a low basal level, even though the endogenous gene is not expressed (17). Expression in the appropriate differentiated cell type is generally only 1 to 2 orders of magnitude above this basal level, whereas the normal differential expression in animals is much greater. Thus, parts of the regulatory mechanism responsible for rigorous, cell-specific expression may be overlooked.

A second approach (Figure 5) is to introduce cloned genes into all cells of an animal by microinjection into fertilized eggs to create *transgenic* animals (18, 19). A major advantage of this method is the ability to assay

for expression in many tissues of an animal. We have introduced the rat elastase I gene into mice to determine whether expression of a transgene can occur in the proper tissue-specific manner.

To introduce the elastase I gene into mice, it was necessary to reassemble the gene by cloning into pBR 322 (20). Since regulatory elements may reside within, nearby or even far outside the structural genes they control, extensive 5'- and 3'-flanking sequences were included in addition to the structural gene. The reassembled gene spanned the 24 kb of genomic sequences shown in Figure 4, with the exception of the 1.5 kb EcoRI fragment within intron 6. Approximately 250 gene copies in 2 picoliters were injected (19) into the male pronucleus of fertilized mouse eggs (Figure 5). Generally, 80 to 90% of microinjected eggs survive. The eggs then were implanted into to oviducts of foster mother mice. About 50% of the implanted eggs develop normally. Generally 15 to 30% of the pups derived from the microinjected eggs have the foreign gene integrated into their genome. To identify pups that have acquired the rat gene, DNA was isolated from the distal 2 cm of the tail of each pup, spotted onto nitrocellulose and hybridized with a probe derived from the 3'-untranslated region of the rat elastase I mRNA. Under stringent hybridization conditions, this hybridization probe does not cross-hybridize with mouse elastase I sequences, and therefore can be used to selectively detect rat elastase I genes (or mRNA) even in the presence of endogenous mouse elastase I genes (or mRNA). In four microinjection experiments with the elastase I gene, 7 mouse pups from a total of 37 born had acquired the rat elastase I gene integrated into the genome. The quantification of integrated genes from tail DNA for 5 of the original 7 transgenic mice is shown in
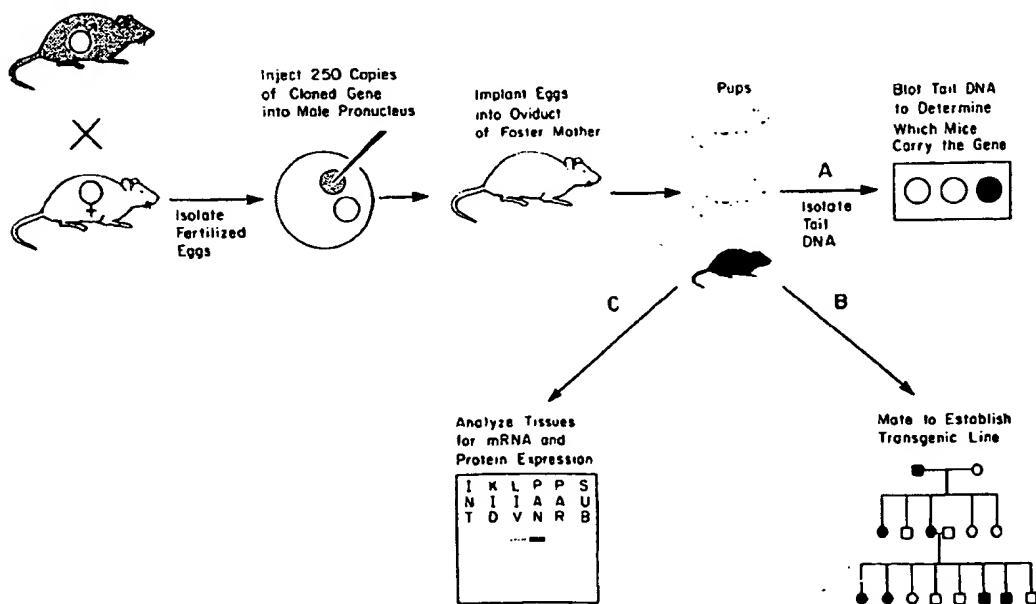
FIG. 5. The production of transgenic mice bearing foreign genes integrated into genomic DNA.

Figure 6. The number of integrated genes varied between 2 and 120 copies among the original transgenic mice.

A number of important parameters concerning the integration of the microinjected gene into the mouse genome cannot be controlled (19). (i) The number of integrated genes varies between transgenic mice, from a fragment of a single gene to several hundred gene copies per cell. The integration of multiple gene copies generally occurs at a single genomic location (occasionally animals with two independent integration sites are found) with the copies linked tandemly in a head-to-tail array. (ii) Integration does not occur preferentially at the position of the endogenous gene. Rather, insertion, if not random, can occur at a large number of sites. (iii) The timing of the integration event may vary. If integration occurs before the first egg cleavage, all cells of the animal carry the genes; integration at later times may limit the introduced gene to only some cells of the animal (mosaicism).

The number of independent integration loci for each transgenic mouse can be determined by following the segregation of the introduced genes in the progeny derived from mating with normal mice. Since transgenic mice are hemizygous for the introduced genes, 50% of the progeny of a single locus transgenic mouse inherit the parental number of gene copies. Greater than 50% of the progeny inherit genes from a transgenic parent with multiple, unlinked loci of introduced genes; the number of genes inherited is characteristic of an individual locus or combination of loci. Fewer than 50% of the progeny inherit the genes if the founding transgenic mouse is mosaic; progeny that inherit the genes have a higher copy number than the apparent copy number of the mosaic parent. Mating experiments with the first five transgenic mice bearing the rat elastase I gene demonstrated the presence of three kinds of integration classes (20). Transgenic Mice 13-4, 6-3 and 9-4 had 2, 7 and 9 gene copies, respectively, integrated at single loci (Figure 6). Mouse 13-1 had 100 gene copies divided into an 80 copy locus and a 20 copy locus. Mouse 22-5 with an apparent copy number of 120 was a mosaic: fewer than 50% of the progeny inherited the rat genes; those that did, inherited twice the parental number.

EXPRESSION OF THE RAT ELASTASE I GENE IN TRANSGENIC MICE

To determine whether the introduced rat genes were expressed, and if so whether expression was pancreas-

specific, we assayed rat elastase I mRNA levels in eight transgenic mouse tissues, including the pancreas (20). Equal amounts of total RNA from each tissue were resolved by electrophoresis in agarose gels containing the denaturant methylmercury hydroxide, transferred and covalently bound to diazophenylthioether paper and hybridized with the rat-specific hybridization probe derived from the 3'-untranslated region of the elastase I mRNA
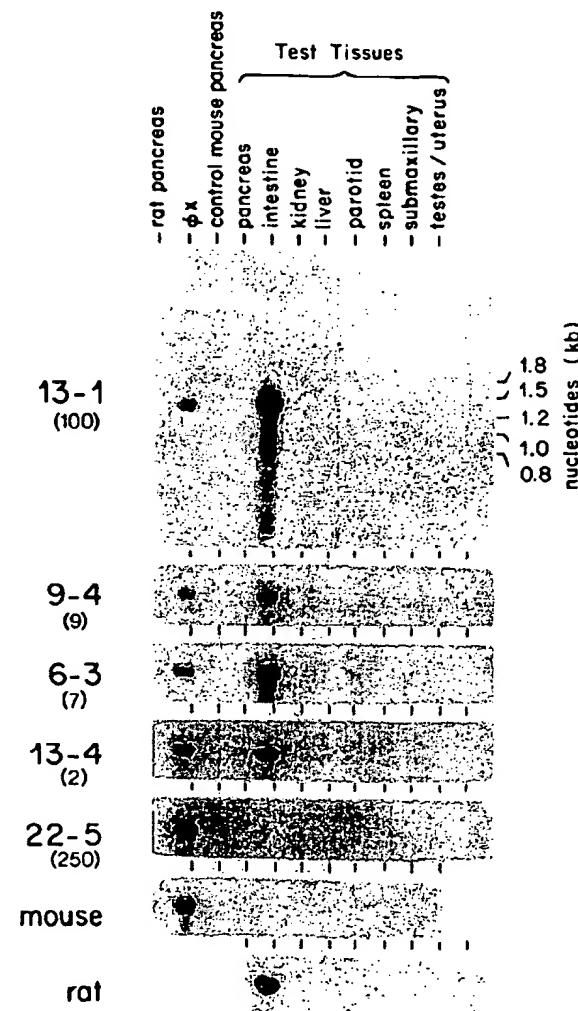
Fig. 7. Northern blot analysis of rat elastase I mRNA expression in transgenic mice. Total RNA was isolated from the tissues listed at the top by the guanidine thiocyanate procedure of Chirgwin et al. (6). Ten micrograms of total RNA from each tissue were resolved by electrophoresis in 1.5% agarose gels containing methyl mercury hydroxide (31), transferred (33) and covalently bound to diazophenylthioether paper (34) and hybridized with a rat-specific single-stranded DNA probe derived from the 3'-untranslated region of the rat elastase I mRNA (20). The number of rat gene copies per cell is given in parentheses for each transgenic mouse. Each blot also contained 10μg of normal rat pancreatic RNA (first lane) to show the amount and size of authentic rat elastase I mRNA in normal rat pancreas. The absence of hybridization to mouse elastase mRNA present within the mouse pancreatic RNA sample on each blot (third lane) demonstrated the species specificity of the probe.

Fig. 6. Copy number of the rat elastase I gene in transgenic mice. Total nucleic acid was isolated from tail pieces of a normal mouse, rat and five of the original transgenic mice. Five micrograms of tail DNA from each animal were spotted onto nitrocellulose and hybridized with a ³²P-labeled, rat-specific, single-stranded elastase I probe (20). The numbers of gene copies per cell, based upon ³²P-cpm hybridized relative to the rat DNA, are shown.

(Figure 7). In 4 of the first 5 animals tested, expression was indeed specific to the pancreas. Mice 13-1, 9-4, 6-3 and 13-4 had pancreatic levels of the rat message equal to or greater than the normal rat level. Expression in other tissues was not detectable by this analysis, with the exception of significant, but low, mRNA levels in the spleens of Mice 13-1, 9-4 and 6-3, and in the liver of Mouse 6-3. However, the level of this inappropriate expression was at least 2 orders of magnitude below pancreatic levels.

Expression in Mouse 22-5 was below the level of detection by this Northern blot analysis for all tissues examined. More sensitive solution hybridization revealed rat elastase I mRNA in the pancreas at about 0.5% of the normal rat level, and levels in nonpancreatic tissues were several-fold lower. Thus, some pancreas-specific expression was observed, but at an exceeding low level. Since this transgenic mouse was mosaic, one likely explanation for the lack of pancreatic expression is an extreme mosaicism for the pancreas, such that the rat genes were present in very few pancreatic acinar cells. Consistent with this explanation, transgenic progeny derived from this founding mouse had high levels of rat elastase I mRNA in the pancreas (see Table 2, discussed below). Moreover, the expression was limited to the pancreas. The appearance of high, pancreas-specific expression in transgenic progeny of Mouse 22-5 indicated that the chromosomal site of integration was not responsible for the lack of expression.

The size of the rat elastase I mRNA in the pancreas of transgenic mice was indistinguishable from that of authentic rat elastase I mRNA (Figure 7). This indicated that initiation of transcription of the introduced genes and polyadenylation and splicing of the transcripts were correct. Moreover, accurate synthesis of the rat elastase I isozyme indicated that the entire amino acid coding region of the mRNA was intact and also correctly spliced (see Figure 9, discussed later).

The extremely high pancreatic level of the rat elastase I mRNA can be verified directly by examining the polyadenylated RNA population by agarose gel electrophoresis. Figure 8 displays polyadenylated RNAs isolated from the pancreases of a normal mouse, transgenic

Pancreatic mRNAs
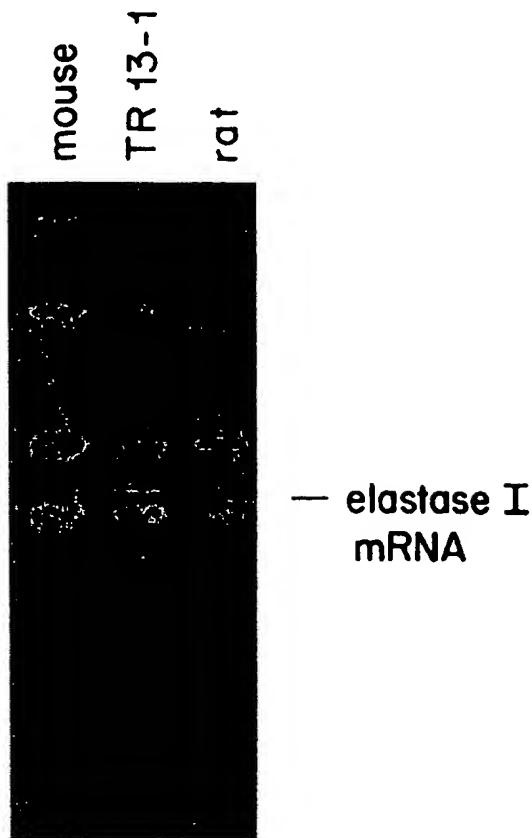
— elastase I
mRNA

FIG. 8. Direct visualization of rat elastase I mRNA in polyadenylated RNA isolated from the pancreas of transgenic Mouse 13-1 (with 100 rat elastase I gene copies). Polyadenylated RNAs isolated from the pancreas of a control mouse, of transgenic Mouse 13-1, and a normal rat were resolved by electrophoresis in a 1.5% agarose gel containing methyl mercury hydroxide and stained with ethidium bromide. The position of migration of elastase I mRNA is noted.

TABLE 2. Rat elastase I mRNA levels in tissues of transgenic mice

Animal	Genes/cell	Rat Elastase I mRNAs per cell			Ratio pancreas:kidney	Ratio pancreas:liver
		Pancreas	Kidney	Liver		
Rat	2	10,000	4	10	2,500	1,000
13-4	2	10,000	<0.3	<1	>33,000	>10,000
6-3	7	45,000	<1	300	>45,000	150
9-4	9	18,000	<0.1	<0.6	>180,000	>30,000
13-1-12	20	45,000	<0.1	6	>450,000	7,500
19-2-22	40	48,000	ND	<1	—	>48,000
13-1-4	80	43,000	<0.1	ND	>430,000	—
13-1	100	120,000	<0.2	3	>600,000	40,000
22-5-5	250	32,000	<1	<2	>32,000	>16,000

"<" indicates that no increase over the background level of detection was observed. The detection level for a given tissue depended upon the amount of RNA available for analysis. ND = not determined.

Mouse 13-1 with 100 rat elastase I gene copies and a normal rat. The level of elastase I mRNA in mouse and rat pancreas is approximately 1% of the total polyadenylated RNA. The level in the RNA from the transgenic mouse was increased strikingly, to become the dominant mRNA (approximately 10% of total). Selective hybridization with the rat-specific elastase I hybridization probe verified that the mRNA was derived from the rat genes and not from augmented expression of the endogenous mouse elastase I genes (20).

SYNTHESIS AND SECRETON OF THE RAT ELASTASE I PROTEIN IN TRANSGENIC MOUSE PANCREAS

Progeny of transgenic mice that inherited the introduced elastase I genes generally expressed them in the pancreas-specific manner of their parent. All transgenic progeny maintain high pancreatic and low-to-undetectable nonpancreatic levels of rat elastase I mRNA.

To demonstrate the correct synthesis of rat elastase I in transgenic mice, pancreatic lobules were dissected from the pancreases of a transgenic progeny mouse derived from Mouse 13-1 and cultured to radiolabel newly synthesized proteins. The labeled proteins were examined by two-dimensional gel electrophoresis and compared to the proteins synthesized by normal rat and mouse pancreas (Figure 9). The presence of a few highly labeled proteins illustrates the dedication of the pancreas to the synthesis of a few enzymes for export. Three isozymes of elastase I (labeled R and M) were synthesized. Their identity was proven by the selective immunoprecipitation of these three proteins by antisera prepared against purified elastase I. Two of the isozymes (labeled M) are from two mouse alleles of the elastase I gene. The third isozyme (labeled R), synthesized at a similar level to the mouse isozymes, is the rat enzyme. A similar analysis of the proteins secreted by these lobules during the 6-hr incubation showed that the rat protein is also secreted at a high level and in a normal fashion. Thus, all aspects of expression of the introduced rat genes (transcription, processing of the mRNA precursor, translation of the mature mRNA and secretion of the mature protein) appear normal.

HOW SELECTIVE IS THE EXPRESSION OF THE RAT ELASTASE I GENES IN MICE?

We assayed the pancreatic levels of the rat elastase I mRNA in eight transgenic mouse lines by solution hybridization (Table 2) to more accurately compare with normal mRNA levels. The number of rat elastase I gene copies in these lines ranged from 2 to 250. Mice 13-1-12, 19-2-22, 13-1-4 and 22-5-5 were the first generation progeny of founding transgenic mice. Mice 13-1-12 and 13-1-4 have inherited the 20 copy and 80 copy locus, respectively, of the dual locus 13-1 parent. Mouse 22-5-5 is an example of the appearance of high pancreas-specific expression in the progeny of the nonexpression mosaic Mouse 22-5. Each transgenic line listed (with the exception of Mouse 13-1, which has two integration sites) represents an independent integration event of rat elastase I genes in the mouse genome.

Each of these eight integration sites supports high,
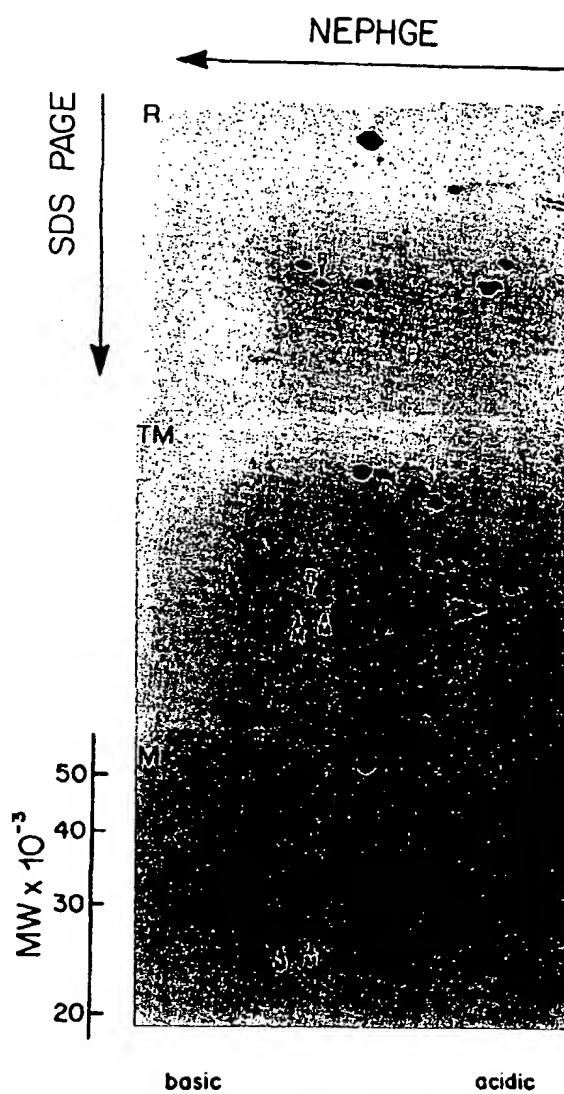
FIG. 9. Synthesis of the elastase I protein at high levels by the pancreas of transgenic mice. Pancreatic lobules were dissected (35) from the pancreas of a normal rat (Panel R), transgenic mouse 13-1-10 (a first generation progeny of founder mouse 13-1) (Panel TM) and a normal mouse (Panel M). The lobules were cultured (2) for 6 hr in the presence of [³H]leucine to radiolabel newly synthesized proteins. This transgenic mouse had approximately 100 copies of the rat gene per cell and 25,000 rat elastase I mRNA molecules per pancreatic cell. The labeled proteins within the tissue lobules were resolved by two-dimensional nonequilibrium pH gradient electrophoresis (36) and autoradiographed. Mouse and rat elastase I isozymes were identified by immunoprecipitation with monospecific antibody against purified rat elastase I (a gift from C. Largman.).

pancreas-specific expression of the foreign rat genes; therefore, appropriate pancreatic expression is essentially independent of chromosomal position. Mouse 13-4, with two rat gene copies per cell, has an mRNA level equivalent to normal rat levels, as if each copy of the rat gene, present in a foreign genome at a novel location, is

expressed as efficiently as a normal elastase I gene in the rat genome at its normal location. The most prodigious producer of rat elastase mRNA is Mouse 13-1, with 120,000 mRNAs per average pancreatic cell. The high pancreatic levels of rat elastase I mRNA in Progeny 13-1-12 and 13-1-4 demonstrate that each of the loci in the original transgenic Mouse 13-1 is active.

The high pancreatic level of rat elastase I mRNA in transgenic mice is more than just a dramatic result: it permits a rigorous test of tissue-specific expression of the introduced genes. Messenger RNA levels in the pancreas and two representative nonpancreatic tissues (kidney and liver) of the eight transgenic mouse lines were compared to determine whether the differential expression of the transgenes was of the same magnitude as the normal endogenous genes (Table 2). Indeed, expression in nonpancreatic tissues of transgenic animals was often undetectable (fewer than 2 mRNAs per cell). The highest nonpancreatic expression was in the liver of Mouse 6-3 with 300 copies per cell, still 150-fold lower than expression in the pancreas of this animal. Differential expression in the pancreas and kidney of mouse 13-1 was greater than 600,000-fold; therefore, even as many as 100 copies of the rat gene integrated at two different sites in the foreign mouse genome were rigorously controlled.

THE LOCATION OF PANCREAS-SPECIFIC REGULATORY SEQUENCES

The recurrent, appropriate pancreatic expression of the rat elastase I transgenes in mice suggested that it should be feasible to identify tissue-specific regulatory sequences by trimming the gene prior to introducing it into mice and assaying for pancreatic expression. Furthermore, results from even a few transgenic animals should be significant. Figure 10 summarizes the results of gene trimming experiments (21). Removing 7 kb of upstream (5') flanking sequences to leave only 205 base pairs upstream of the elastase I structural gene does not

eliminate pancreas-specific expression. The four mice bearing this trimmed gene had an average of 3,400 rat elastase I mRNAs per cell; expression in seven nonpancreatic tissues was undetectable.

In a series of fusion gene experiments (Figure 10), progressively shorter lengths of elastase I 5'-flanking sequences were linked to the structural gene for human growth hormone (hGH) (21). The fusion gene with 4.2 kb of flanking sequence linked to the hGH gene directed high, pancreas-specific expression of hGH mRNA in 6 of 8 transgenic mice. This result, coupled with the result of the trimmed gene experiment, indicated the presence of either: (a) at least two pancreatic regulatory elements, one in the upstream region and one in the body of the elastase I gene or downstream flanking region or (b) a single regulatory element within the only region common to the two gene constructs—the elastase gene region between −205 and +8. To test these two possibilities directly, only the elastase I region −205 to +8 was joined to the hGH gene. In 4 of 6 transgenic animals bearing this fusion gene, hGH mRNA was found at high levels in the pancreas and not in other tissues.

The expression of these fusion gene constructs yields a hybrid mRNA containing only the first eight nucleotides of the elastase I mRNA linked to the hGH mRNA missing its first two nucleotides. The size of the mRNA produced in the pancreas was indistinguishable from authentic hGH mRNA (21). This result indicated that the hGH mRNA was transcribed and spliced properly. This short elastase I gene region conferred transcriptional specificity in transgenic mice bearing the elastase-hGH fusion gene, since pancreatic nuclei, but not hepatic nuclei, synthesized hGH RNA (22). Moreover, immunohistochemistry demonstrated that hGH protein was present in the acinar cells but not in endocrine or connective tissue cells of the pancreas. Thus, expression was directed to the appropriate cell type within the tissue.

These fusion gene experiments permit a complete an-
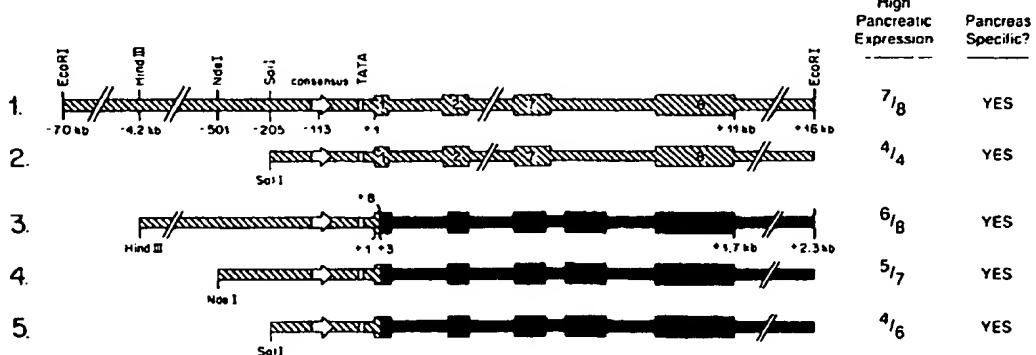
FIG. 10. A pancreas-specific regulatory element is located near the transcription start site of the elastase I gene. The cloned rat elastase I gene was modified prior to microinjection into mouse eggs. (Construct 1) Unmodified gene comprising 7 kb 5'-flanking, 11 kb of exon and intron sequences and 5 kb of 3'-flanking sequences. (Construct 2) All but 205 nucleotides upstream from the start of transcription deleted. (Construct 3) Fusion of the 5'-flanking region of the elastase I gene from −4,200 to +8 nucleotides to the hGH structural gene at +3. (Construct 4) Fusion of −500 to +8 of elastase I to hGH at +3. (Construct 5) Fusion of −205 to +8 of elastase I to hGH at +3. The number of transgenic mice with pancreatic expression of rat elastase I mRNA out of the total number tested is given at the right. Expression varied between 1,200 and 120,000 mRNAs per cell, depending upon the gene construct and the individual animal. Pancreas specificity was indicated by the absence of detectable elastase I mRNA in several other tissues (less than 10 rat elastase I mRNAs per cell) (21).

imal assay for expression in nonpancreatic tissues. Although synthesis and secretion of hGH by the acinar cells of the pancreas of transgenic mice would result in the degradation of the hormone in the intestine and not affect growth, ectopic expression of hGH would dramatically increase growth (23), even at low levels (24). However, none of the transgenic mice with the elastase I-hGH fusion gene grow detectably larger than their normal litter mates. This absence of enhanced growth in animals bearing elastase I-hGH fusion genes also indicates that expression does not occur in nonpancreatic tissues, including far more tissues than could be dissected and analyzed for mRNA levels by hybridization. Any cell type in the animal that secretes into a compartment that has access to the circulation can be excluded as having significant expression of the elastase-hGH transgene.

NATURE OF THE PANCREATIC REGULATORY SEQUENCE

The −205 to +8 region of the elastase I gene that is sufficient to direct pancreas-specific expression contains the start site of transcription, the promoter and a 25-nucleotide sequence recognizably conserved among several pancreas-specific serine protease genes (Figure 11). The conserved sequences is likely an important component of the regulatory region. It is included within a region necessary for selective expression of chymotrypsin gene constructs in pancreatic acinar cells in culture (16). Octanucleotide sequences within the conserved regions resemble the core sequence of the SV40 enhancer (15). Enhancers have the remarkable ability to stimulate transcription over long distances of more than 1 kb, in either orientation and either upstream or downstream of the start site of transcription (25). Our recent results indicate that the elastase I regulatory sequence exhibits enhancer-like properties in a pancreas-specific manner when tested in transgenic mice.

Cis-acting regulatory information sufficient for pancreas-specific transcription in animals is contained within the short elastase I gene sequence between −205 and +8. Moreover, the transcription of the elastase-hGH fusion gene correlates with a pancreas-specific DNase I hypersensitive site within the elastase I regulatory region (22). DNase I hypersensitive sites are indicative of changes in chromatin structure associated with active transcription. If pancreas-specific trans-acting factors must bind to activate transcription, then this 213 bp elastase I gene fragment contains all the necessary information for binding. If pancreas-specific alterations in chromatin are necessary to activate the gene, that infor-

mation also must be contained within this narrow upstream gene region. Furthermore, if there are important constraints on nuclear architecture, such as positioning the active gene at or near the nuclear membrane or in association with the nuclear matrix in a pancreas-specific manner, then this fragment must contain that information as well. Elucidation of the role of these molecular processes remains an important goal in understanding the mechanism of pancreatic expression mediated by the elastase I regulatory element.

PROSPECTS

The elastase I regulatory domain is well-defined, dominant, cis-acting and capable of directing pancreas-specific expression not only of its cognate structural gene, but also unrelated genes such as hGH when linked by recombinant DNA techniques. The directed expression of a transgene permits an experimental approach to defining gene functions in animals not previously possible. The tissue-specific expression of a transgene permits the selective analysis of the effects of aberrant expression in a single cell type without pleiotropic effects on the development of the transgenic embryo or on the physiology of the transgenic adult. One dramatic example is the tissue-specific oncogenesis directed by the SV40 T-antigen gene when linked to tissue-specific regulatory sequences. Transgenic mice bearing the T-antigen gene with its normal SV40 promoter and regulatory elements develop tumors selectively of the choroid plexus, apparently mirroring the inherent trophic properties of the SV40 virus (26, 27). Replacement of the SV40 control sequences with those of elastase I redirects T-antigen expression and tumor formation selectively to pancreatic acinar cells (22). Transgenic mice with the elastase-T-antigen fusion gene invariably develop pancreatic acinar cell carcinomas in a predictable manner and provide a valuable paradigm of tumorigenesis.

The elastase regulatory region is clearly not unique; other tissue-specific genes also have regulatory regions seemingly indifferent to the nature of the structural gene whose expression they can be made to control. For example, the insulin regulatory region directs T-antigen expression and tumor formation selectively to pancreatic endocrine cells (28). Immunoglobulin regulatory elements similarly direct c-myc oncogene expression and tumor formation to lymphocytes (29). The identification of regulatory sequences for other tissues (such as those of the liver or gut) is likely and would extend this kind of analysis to specific functions of other cell types. For example, directed expression could test the role of a structural protein in the morphogenesis of a tissue, or the role of a regulatory enzyme like a protein kinase on the control of a cell function such as secretion. Thus, continued characterization of cell-specific regulatory sequences should permit the analysis of gene function in a direct manner not previously possible.

FIG. 11. Sequences within the 5'-flanking region of the rat elastase I gene also conserved within the 5'-flanking regions of the chymotrypsin B (12), trypsins I and II (13) and elastase II (15) genes. Numbering begins (+1) at the start site of elastase gene transcription. The conserved region between −120 and −98 conforms to a consensus sequence derived from all five of the pancreas-specific serine protease genes.

Galvin Swift, Jenny Stary, Steve Lowther, Jean Charles Dagorn, Pat Ashley, Charles Craik and Bill Rutter. In addition, we were very fortunate to have the opportunity to collaborate with Ralph Brinster, Bob Hammer and Myrna Trumbauer to produce and analyze transgenic mice. The analysis of expression in transgenic animals was a shared effort with Galvin Swift, Brian Davis, Dave Ornitz and Richard Palmiter. This report is dedicated to the memory of Steve Lowther.

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The Hypogonadal Mouse: Reproductive Functions Restored by Gene Therapy

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The Hypogonadal Mouse: Reproductive Functions Restored by Gene Therapy

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The hypogonadal (*hpg*) mouse lacks a complete gonadotropin-releasing hormone (GnRH) gene and consequently cannot reproduce. Introduction of an intact GnRH gene into the genome of these mutant mice resulted in complete reversal of the hypogonadal phenotype. Transgenic *hpg/hpg* homozygotes of both sexes were capable of mating and producing offspring. Pituitary and serum concentrations of luteinizing hormone, follicle-stimulating hormone, and prolactin were restored to those of normal animals. Immunocytochemistry and in situ hybridization showed that GnRH expression was restored in the appropriate hypothalamic neurons of the transgenic *hpg* animals, an indication of neural-specific expression of the introduced gene.

MICE HOMOZYGOUS FOR THE HYPOGONADAL (*hpg*) MUTA-tion are sexually immature and have arrested germ cell development (*1*). Immunocytochemical and radioimmunoassay measurements (*1, 2*) have shown that these mice lack detectable levels of hypothalamic gonadotropin-releasing hormone (GnRH), leading to low levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

As described in the accompanying article (*3*), the genetic basis for this mutation is a deletion of at least 33.5 kilobases encompassing the distal half of the gene for the common biosynthetic precursor of GnRH and GnRH-associated peptide (GAP) (*4, 5*). This deletion does not seem to affect the number and location of the GnRH neurons in the *hpg* hypothalamus since messenger RNA (mRNA) transcripts from the truncated *hpg* gene can be detected by in situ hybridization in the expected locations of the *hpg* brain (*3*). The normal architecture of GnRH neurons and neuronal circuitry necessary to control the pulsatility and correct amplitude of GnRH and GAP (*6*) output may therefore not be directly affected by the *hpg* mutation.

To try and restore a functional hypothalamic-pituitary-gonadal axis, we introduced a DNA fragment containing the mouse GnRH gene with 5 kb of 5' flanking and 3.5 kb of 3' flanking sequences into a wild-type mouse. By a series of matings with *hpg/*+ heterozygotes, the transgene was introduced into a *hpg/hpg* homozygous background. Transgenic *hpg/hpg* homozygotes displayed tissue-specific expression of the GnRH transgene, they reached full sexual maturity, and were capable of mating and raising healthy litters.

Introduction of GnRH-GAP gene into *hpg/hpg* homozygotes. Since homozygous *hpg/hpg* mice are sterile, we introduced the GnRH-GAP gene into the *hpg/hpg* homozygous background as outlined in Fig. 1A. The genotype of each animal from this series of

matings was established by Southern genomic DNA hybridization with probe 2 (Fig. 1, B and D). This Bam HI–Eco RI fragment, which encompasses the 3' breakpoint of the *hpg* deletion and thus does not detect the presence of the transgene, could distinguish between genomic DNA from wild-type, *hpg/*+, and *hpg/hpg* mice (Fig. 1D). Sequential hybridization of the same genomic DNA's with probe 1, which is specific for intron B and exon III of mouse GnRH gene, was used to establish which of the mice contained the introduced transgene (Fig. 1, B and C).

Approximately 200 copies of a gel-purified 13.5-kb Sal I–BstE II fragment (Fig. 1B) containing the mouse GnRH gene flanked by 5 kb of upstream and 3.5 kb of downstream sequences were microinjected into 250 fertilized wild-type mouse eggs (C57BL/6J × SJL). A total of 27 pups were born, of which two were shown to be transgenic, each containing more than 20 copies of the transgene. To introduce the GnRH gene into a *hpg/*+ heterozygous background, transgenic mice A and B were backcrossed to *hpg/*+ heterozygotes (Fig. 1A). Of the 18 offspring of mouse B, 14 inherited the transgene. Only 6 of the 30 offspring of transgenic mouse A were transgenic. Although this result suggested that the germ line of mouse A was mosaic for the transgene, an identical copy number of the transgene was maintained in the offspring of this mouse. As was expected, approximately half of the offspring from the above crosses were *hpg/*+ heterozygotes.

Mice identified as transgenic *hpg/*+ heterozygotes from both families were crossed with either another transgenic *hpg/*+ heterozygote from the same family or a nontransgenic *hpg/*+ heterozygote. Approximately one-quarter of the offspring from these crosses were *hpg/hpg* homozygotes (see Fig. 1D). Of a total of 12 *hpg/hpg* homozygotes identified in family B, seven were also transgenic, two being males (B-41, B-61) and five females (B-26, B-50, B-66, B-68, B-69). A surgical examination of the transgenic *hpg/hpg* homozygotes and their five nontransgenic *hpg/hpg* homozygous littermates revealed that the transgenic mice had well-developed gonads and accessory sexual glands compared to their *hpg* littermates whose gonadal tissue was developmentally arrested at the prepubertal stage. The same results were observed with the A family, in which each of seven transgenic *hpg/hpg* homozygotes was sexually mature.

Mating and pregnancy can occur in transgenic *hpg* animals. To investigate whether the transgenic *hpg* males were capable of mating and fathering offspring, two transgenic *hpg* males (B-41 and B-61) were each paired with a normal female. The females found to have vaginal plugs (a sign of successful copulation) were killed and most of their eggs were fertilized as seen by microscopic examination. The fertilized eggs were transplanted into a pseudopregnant female, and

approximately 12 days after transplantation the foster female was killed and genomic DNA was extracted from each of the embryos. Results of a Southern analysis of this DNA showed that the nine embryos fathered by mouse B-41, and ten embryos fathered by mouse B-61 were all heterozygous for the *hpg* allele, thus confirming our assignment of B-41 and B-61 as *hpg/hpg* homozygotes (Fig. 1E). This experiment demonstrated that the transgenic *hpg* males were capable of correct mating behavior and of fertilizing eggs.

Transgenic *hpg* females had also regained their full reproductive ability, since three such females from the B family when paired with transgenic *hpg/+* heterozygote males became pregnant and gave birth to healthy litters of between 6 and 11 mice. Analogous experiments with mice from the A family showed that the males and females were reproductively competent. Offspring from such crosses were genotyped and those identified as transgenic *hpg/hpg* homozygotes were killed for detailed endocrinological and expression studies.
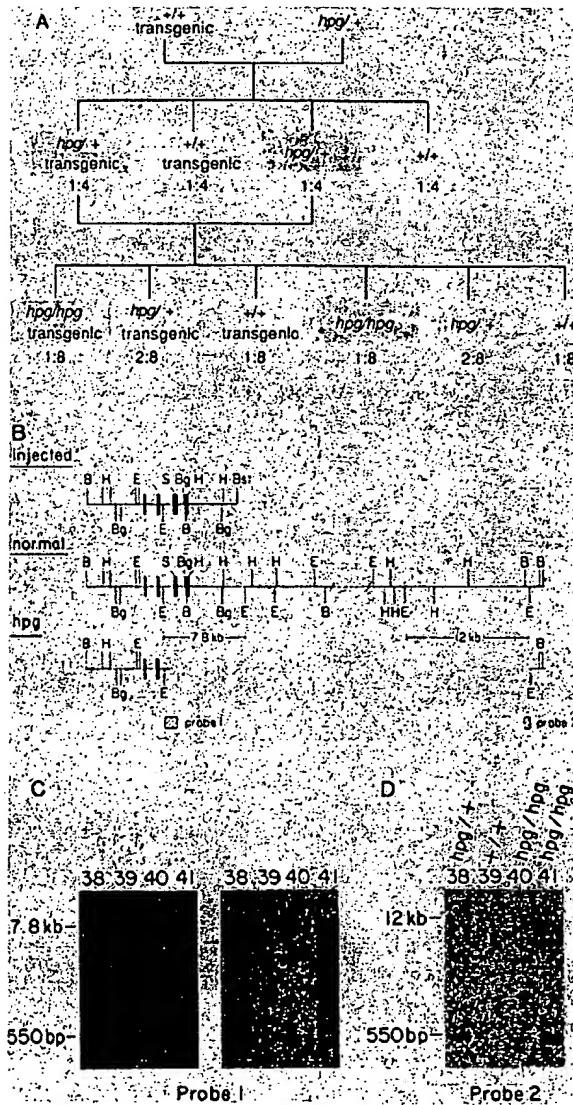
Gonadal development in transgenic *hpg/hpg* homozygotes. To investigate the extent of gonadal development in the transgenic *hpg* mice the following study was performed. From the B family, five transgenic *hpg/hpg* homozygous females and four transgenic *hpg/hpg* homozygous males approximately 12 weeks old were killed along with 10-week-old wild-type male and female mice and 8-week-old *hpg/hpg* homozygotes as controls. The testes, seminal vesicles, preputial glands, and adrenals were surgically removed from the male mice and weighed. Similarly, the ovaries, uteri, and adrenals were removed from the females and their weight was determined (Fig. 2A). In the transgenic *hpg/hpg* homozygous males the testes, seminal vesicles, and preputial glands had developed to normal size. Similar results are seen on comparison of normal ovarian and uterine weights in normal and transgenic *hpg/hpg* homozygous females. The weights of the ovaries and uteri from transgenic *hpg* females were slightly higher, but not significantly, than those of the normal females. The weights of the adrenals of the three groups of male and

Fig. 1. Generation and identification of transgenic *hpg/hpg* mice. (A) Schematic of protocol for generation of *hpg/hpg* homozygous mice carrying the GnRH-GAP transgene. The 13.5-kb Sal I–BstE II GnRH-GAP gene fragment was generated from a Bam HI–Eco RI subclone of the entire insert of the lambda phage λmGP-1 (3). The Sal I restriction site is adjacent to the Bam HI site of the plasmid polylinker sequence. This fragment was gel purified and injected (21) into a pronucleus of one-cell fertilized mouse eggs. The eggs were derived by mating C57BL/6J females and SJL males. Transgenic mice were identified as described above and are indicated as being wild type at the hypogonadal locus (+/+) and heterozygous for the GnRH transgene (transgenic). The transgenic mice identified were back-crossed to nontransgenic mice heterozygous for the *hpg* allele (*hpg/+*). Mice resulting from this mating that were heterozygous *hpg/+* and transgenic were backcrossed to *hpg/+* heterozygous mates. Among the offspring of this mating were animals that were homozygous *hpg/hpg*, approximately half of these were transgenic and half were nontransgenic. The indicated genotypes are expected to occur at a frequency represented by the ratios shown below each. (B) Restriction enzyme map of the GnRH genes and probes used. The normal and mutant alleles of the GnRH gene are more fully described in (3). The injected gene was a Sal I–BstE II fragment derived from a subclone of the normal GnRH gene (3). Probe 1 is a 767-bp Eco RI–Sac I fragment (hatched square) that contains sequences for a portion of intron B and exon III of the mouse GnRH gene. Probe 2 (hatched oblong) is a 360-bp Bam HI–Eco RI fragment containing the 3′ breakpoint of the *hpg* deletion (3). (C) Four third-generation mice (mouse 38 to mouse 41) from the B family were analyzed for the presence of the GnRH-GAP transgene by Southern analysis with probe 1 hybridized to the same filter used in (D) (22). At short exposures (right panel, 2 hours) only the transgene is detected in mouse 38 and mouse 41. At longer exposures (left panel, 20 hours) the expected endogenous 7.8-kb band is seen in mouse 39. As expected from the results seen in panel C, this 7.8-kb band is not seen in mouse 40. (D) The same four mice, as in (C), were tested for its genotype at the hypogonadal locus by Southern analysis with probe 2. The DNA was extracted from the tails (23), digested with Eco RI, purified by electrophoresis through 1.2 percent agarose, transferred to a nylon membrane, and hybridized with probe 2 (22). The mutant GnRH gene *hpg* allele is revealed by a band at 550 bp and the normal gene by a band at 12.0 kb. Thus mouse 38 is an *hpg/+* heterozygote, mouse 39 is wild type (+/+), and mouse 40 and mouse 41 are *hpg/hpg* homozygotes. (E) Breeding analysis of a transgenic *hpg/hpg* animal. Mouse B-41, identified as being transgenic *hpg/hpg* male on the basis of Southern analysis, was crossed to a wild-type mouse. The panels show the results of Southern analysis of mouse 41, its wild-type mate, and the resulting offspring (mouse 144 to mouse 152) with the use of probe 2.
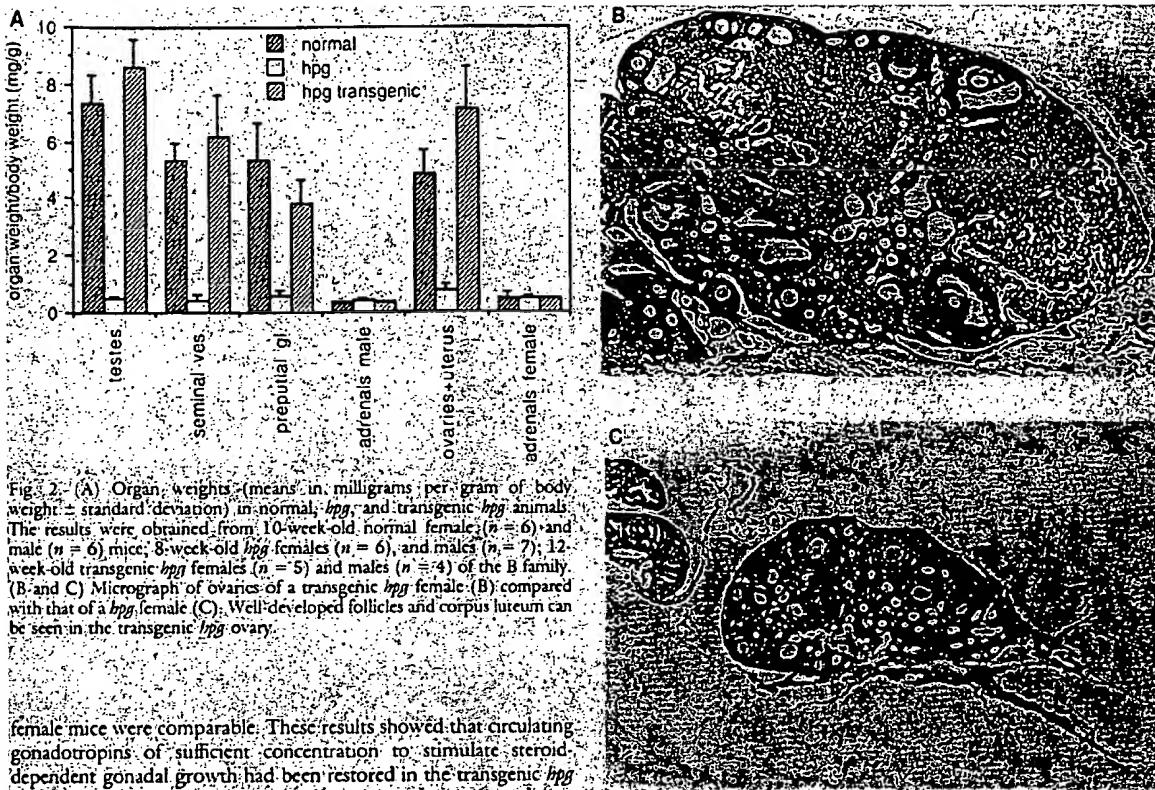
Fig. 2. (A) Organ weights (means in milligrams per gram of body weight ± standard deviation) in normal, hpg, and transgenic hpg animals. The results were obtained from 10-week-old normal females (n = 6) and male (n = 6) mice; 8-week-old hpg females (n = 6), and males (n = 7); 12-week-old transgenic hpg females (n = 5) and males (n = 4) of the B family. (B and C) Micrograph of ovaries of a transgenic hpg female (B) compared with that of a hpg female (C). Well-developed follicles and corpus luteum can be seen in the transgenic hpg ovary.

female mice were comparable. These results showed that circulating gonadotropins of sufficient concentration to stimulate steroid-dependent gonadal growth had been restored in the transgenic hpg mice.

Histological examination of the testes from transgenic hpg/hpg homozygotes showed a normal arrangement of fully developed interstitial tubules, Leydig cells, and Sertoli cells. Examination of histological sections of the ovaries from transgenic hpg/hpg females revealed characteristic follicles at different stages of development and corpora lutea (Fig. 2B), confirming our earlier observations that the females were undergoing normal estrous cycles.

Gonadotropin levels in transgenic hpg/hpg homozygotes. The fact that the transgenic hpg homozygotes had fully developed gonads and accessory glands and produced offspring showed that a normally functioning reproductive endocrine system had been restored in these animals. To confirm this, serum levels and pituitary contents of luteinizing hormone, follicle-stimulating hormone, and prolactin were determined in 12-week-old transgenic hpg mice of the B family. These values were compared with those of hpg/hpg homozygotes (8 weeks of age) and normal controls (10 weeks of age). The results are summarized in Table 1. Serum levels of FSH in transgenic hpg females were essentially identical to normal females and were significantly higher than in hpg/hpg homozygotes. Serum FSH in transgenic hpg males was slightly higher than in the control animals but well within the normal range. FSH levels were also measured in a male transgenic hpg mouse and a female transgenic hpg mouse from the A family and found to be within the normal range.

The pituitary content of FSH in transgenic hpg females was slightly lower than normal controls but significantly higher than hpg homozygotes. In transgenic hpg males and normal mice, pituitary FSH contents did not differ significantly, both being clearly higher than hpg homozygotes. The LH levels in serum (Table 1) of transgenic hpg females and males were somewhat lower than normal levels, yet both were much higher than hpg/hpg homozygotes. Pituitary LH content in transgenic hpg female mice was approxi-

mately 50 percent of that of the normal animals but again much higher than in hpg/hpg homozygotes. In the transgenic hpg male group, pituitary LH content was about 70 percent of the level in normal mice but clearly higher than in hpg/hpg homozygotes.

As shown in Table 1, the pituitary content of prolactin was not significantly different from the normal range in transgenic hpg males and female mice, a result consistent with the prolactin release-inhibiting effects of GAP (5). Serum levels of prolactin were significantly lower in normal and transgenic hpg males than in the hpg males, also supporting the inhibitory effect of GAP. However, in females the opposite was found, that is, normal and transgenic hpg animals had significantly higher circulating levels of prolactin than hpg females. In an independent study, a different result was obtained, namely, that normal animals had higher serum prolactin values than hpg/hpg homozygotes (7). This could be due to age and strain differences between the three groups, cyclicity in the normal and transgenic groups, and the effect of estradiol or other unknown factors that influence the synthesis and secretion of prolactin (8).

Tissue distribution of GnRH and GAP. To examine the specificity of expression of the introduced GnRH-GAP gene in transgenic hpg animals, we analyzed various tissues by radioimmunoassay. Brains of nine transgenic hpg mice (four males and five females from family B) at approximately 12 weeks of age were extracted, and their GnRH and GAP contents determined. Values were compared with those of hpg/hpg homozygotes (8 weeks of age) and normal mice (10 weeks of age). In addition to brain, the following tissues were extracted and assayed for their content of the two peptides from one individual of each group: testes, seminal vesicles, ovaries, uterus, adrenal, liver, kidney, pancreas, thymus, and spleen. GnRH was extracted and determined with a previously

described assay based on a "conformational" antibody, EL-14 (9). This antibody detects only the mature processed form of the decapeptide with blocked terminals. GAP was extracted and assayed (10) with an antibody (56A) produced against residues 20 to 43 of rat GAP. Rat GAP and mouse GAP are essentially identical in the region recognized by this antibody, with only one amino acid change (3, 4). The results of these experiments are summarized in Table 2.

The overall tissue distribution of GnRH immunoreactivity and GAP immunoreactivity in the transgenic *hpg/hpg* homozygotes

resembled that of normal animals. In all tissues found to contain GnRH and GAP, GAP levels on a molar basis were always twofold higher than that of GnRH. This reflects the ratio of precursor to processed hormone, since the GAP antibody can also detect the unprocessed GnRH-GAP precursor (11). Brain and testicular contents of both GnRH and GAP or prohormone in the transgenic *hpg/ hpg* homozygous males were within the range of normal levels as shown in Table 2. The only major difference between normal and transgenic *hpg* males was the presence of very low levels of the two gene products in the livers of the latter. In females, contents of

Sagittal section showing the GnRH immunoreactive perikarya found in the paraventricular nucleus of the transgenic *hpg* mouse (125×). (D) Sagittal section of the preoptic region (B) after in situ hybridization to ²⁵S-labeled GnRH riboprobe showing the distribution of mRNA containing cells (125×, exposure 3 weeks, polarized light-bright field). (E) Sagittal section of the paraventricular nucleus after in situ hybridization showing GnRH mRNA containing neurons (125×, exposure 3 weeks, polarized light and bright field). Sagittal sections taken every 100 μm were stained with antibody DBI (1:2000 dilution) by a modification of the avidin-biotin peroxidase complex method (10). In situ hybridization was performed as described (24), except as follows. Animals were killed, and the brains were fixed by immersion in 4 percent paraformaldehyde and 0.1M sodium phosphate buffer (pH 7.4). Cryostat sections (10 μm) were thaw-mounted on polylysine-coated slides. The sections were treated with proteinase K at 5 μg/ml and hybridized to an SP6 riboprobe (25) generated from a rat GnRH cDNA fragment (4) labeled with [³⁵S]ATP (Amersham), in hybridization buffer containing 50 percent formamide, 0.3M NaCl, 20 mM tris, pH 8.0, 5 mM EDTA, 1× Denhardt solution, 10 percent dextran sulfate, and 10 mM dithiothreitol. After hybridization, the slide preparations were washed twice for 10 minutes in 2× SSC and then treated with ribonuclease A (20 μg/ml) and subsequently washed with 0.2× SSC at 42°C. All washings after the hybridization procedure contained 10 mM β-mercaptoethanol and 1 mM EDTA. The sections were dehydrated and dipped in Kodak NTB2 nuclear emulsion and exposed for 3 weeks at 4°C. The sections were stained with hematoxylin and eosin.

Fig. 3. Immunocytochemical and in situ hybridization localization of GnRH neurons. (A) Sagittal section through the median eminence of a transgenic *hpg* mouse showing normal distribution of GnRH fiber terminals (100×). (B) Sagittal section representative of the GnRH immunoreactive cell bodies found in the preoptic region of the transgenic *hpg* mouse (125×). (C)

GnRH and GAP or prohormone in the central nervous system (CNS) were close to those of normal animals. Gonadal content of the two peptides was slightly less than normal. In the transgenic hpg female a low amount of GnRH and GAP was again detected in the liver. Liver-specific expression of GnRH and GAP was also demonstrated in transgenic hpg animals of the other transgenic line. Because of the sensitivity of the radioimmunoassay, we cannot discount that an equally low level of expression is also occurring in other tissues. Extremely low levels of GnRH and GAP were detected in the kidneys of normal and transgenic hpg males, but only GAP was detected in the kidney of transgenic hpg females. The finding of GnRH and GAP in the kidney may be attributable to the fact that the kidney is the major degradation site for GnRH (12) and presumably for GAP.

Localization of GnRH-GAP neurons in hpg transgenic animals. The distribution and density of cells containing GnRH were compared between normal and transgenic hpg animals of the A and B family by immunocytochemistry and in situ hybridization (Fig.

Table 1. Serum levels and pituitary content of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL) in transgenic hpg/hpg homozygotes (Trans. hpg) compared with normal and hpg control groups. Hormone concentrations were determined by double-antibody radioimmunoassays (RIA) as described (26) using materials provided by the National Hormone and Pituitary Program, NIADDK, for the corresponding rat hormones. These included FSH-RP-2, FSH-I-6, FSH-S-11, LH-RP-2, LH-I-6, LH-S-9, PRL-RP-3, PRL-I-5, and PRL-S-9. The assay sensitivities allowed individual determinations of FSH and PRL concentrations in both serum and pituitary and LH in pituitary but sera from two animals per group had to be pooled to obtain a large enough sample for the LH RIA. There were obvious substantial differences between males and females for pituitary FSH, serum FSH, pituitary LH, and serum prolactin. Hence, these data were analyzed separately for males and females. However, analysis of variance with sex and treatment group indicated no notable differences between males and females for serum LH ($P = 0.1032$) or pituitary prolactin ($P = 0.1862$). Therefore, males and females were combined for the analysis of these data. Two-sample t tests were used to compare each pair of treatment groups. The P values (unadjusted for multiple comparisons) are listed below. These were adjusted for the fact that multiple comparisons were being made. Conservatively, Bonferroni's inequality was used for this adjustment (27). Of all the P values listed, only one changed from being significant ($P = 0.0289$) to nonsignificant as a result of this adjustment. This was in the case of serum FSH between normal males compared to transgenic males, and therefore the difference between these groups is questionable.

Mouse genotype	Serum level (ng/ml ± SD)		Pituitary content (ng/pituitary ± SD)	
	Male	Female	Male	Female
Follicle-stimulating hormone (FSH) *				
Normal	27.957 ±4.809	12.390 ± 4.430	320.87 ± 66.16	44.79 ± 3.97
hpg	3.679 ±1.490	4.906 ± 1.248	32.59 ± 2.67	18.75 ± 0.17
Trans. hpg	38.650 ±6.553	12.332 ± 5.358	332.89 ± 28.59	33.72 ± 1.55
Luteinizing hormone (LH)†				
Normal	0.42 ±0.12	0.48 ± 0.18	1168.36 ± 116.04	299.68 ± 19.89
hpg	<0.05	<0.05.	79.19 ± 4.53	48.52 ± 1.58
Trans. hpg	0.27 ±0.11	0.31 ± 0.09	833.04 ± 41.93	152.15 ± 11.44
Prolactin (PRL)‡				
Normal	0.940 ±0.327	2.970 ± 0.800	3.165 ± 0.665	3.080 ± 0.230
hpg	1.753 ±0.720	1.264 ± 0.461	2.055 ± 0.200	2.195 ± 0.086
Trans. hpg	0.676 ±0.203	2.747 ± 0.267	2.700 ± 0.210	2.765 ± 0.405

*FSH pituitary levels: transgenic hpg females versus normal females ($P < 0.00005$), both versus hpg/hpg homozygotes ($P < 0.00005$). Transgenic hpg males versus normal males ($P = 0.4066$), both versus hpg/hpg homozygotes, significance ($P < 0.00005$). FSH serum levels: transgenic hpg females versus normal females ($P = 0.9898$), normal versus hpg/hpg homozygotes ($P < 0.0089$), transgenic versus hpg/hpg homozygotes ($P = 0.0141$). Transgenic hpg males versus normal males ($P = 0.0289$), both versus hpg/hpg homozygotes, significance ($P < 0.00005$). †LH pituitary levels: transgenic hpg females versus normal females ($P = 0.0002$), both versus hpg/hpg homozygotes ($P < 0.00005$). Transgenic hpg males versus normal males ($P < 0.00005$), both versus hpg/hpg homozygotes, significance ($P < 0.00005$). LH serum levels: transgenic hpg versus normal ($P = 0.00002$), both versus hpg/hpg homozygotes ($P = 0.00005$). ‡PRL pituitary levels: transgenic hpg versus normal ($P = 0.0040$), normal versus hpg/hpg homozygotes ($P < 0.00005$), transgenic versus hpg/hpg homozygotes ($P < 0.0055$). PRL serum levels: transgenic hpg females versus normal females ($P = 0.0002$), both versus hpg/hpg homozygotes ($P = 0.00005$). Transgenic hpg males versus normal males ($P = 0.0084$), both versus hpg/hpg homozygotes, significance ($P < 0.00005$).

Table 2. Tissue contents of GnRH and GAP in normal, hpg/hpg homozygous, and transgenic hpg/hpg homozygous (transgenic hpg) male and female mice. Brains from 10-week-old normal mice ($n = 6$), 8-week-old hpg mice ($n = 6$), and 12-week-old transgenic hpg mice ($n = 5$ females, $n = 4$ males, family B) were removed and quickly frozen in liquid nitrogen. The other tissues indicated were obtained from one mouse in each group. Tissues were homogenized and extracted as described (9, 10) and the supernatants were analyzed by protein assay (28) and GnRH (9) and GAP (10) radioimmunoassays.

Mouse		Tissue content (pg/mg)*						
Genotype	Sex	Brain	Gonads	Adrenals	Liver	Kidneys	Pancreas	Spleen plus thymus
Gonadotropin-releasing hormone (GnRH)								
Normal	Male	41.15 ± 3.86	28.39 ± 4.68	<0.40	<0.40	0.59 ± 0.11	<0.40	<0.40
hpg		<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40
Trans. hpg		35.82 ± 2.89	26.91 ± 4.85	<0.40	1.45 ± 0.13	0.68 ± 0.18	<0.40	<0.40
Normal	Female	26.75 ± 2.17	17.45 ± 1.98	<0.40	<0.40	<0.40	<0.40	<0.40
hpg		<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40
Trans. hpg		19.89 ± 1.86	12.21 ± 0.95	<0.40	0.52 ± 0.11	<0.40	<0.40	<0.40
GnRH-associated peptide (GAP)								
Normal	Male	432.0 ± 88	295.0 ± 16	<10	<10	11.2 ± 3.3	<10	<10
hpg		<10	<10	<10	<10	<10	<10	<10
Trans. hpg		495.0 ± 83	366.0 ± 93	<10	24.0 ± 3.4	15.2 ± 5.4	<10	<10
Normal	Female	516.0 ± 181	368.0 ± 88	<10	<10	<10	<10	<10
hpg		<10	<10	<10	<10	<10	<10	<10
Trans. hpg		385.0 ± 107	184.0 ± 35	<10	15.6 ± 5.5	27.0 ± 6.9	<10	<10

*Picograms per milligram of protein, means ±SD.

3). Transgenic *hpg/hpg* homozygous mice of both sexes showed immunoreactive cells in the medial preoptic and septal nuclei, superchiasmatic region of the hypothalamus, the bed nucleus of the stria terminalis, the olfactory bulbs, and paraolfactory regions. The same distribution and approximately the same number of cells was seen after in situ hybridization of serial sections to a ³⁵S-labeled GnRH-GAP probe. An additional group of cells was noted in the paraventricular nucleus (PVN) of the thalamus in transgenic animals that was not seen in normal controls (Fig. 3, C and E). Unlike the typical GnRH neurons observed in the hypothalamus, the GnRH-containing PVN neurons did not display any immunoreactive processes (Fig. 3C). Immunoreactive cells outnumbered those found in the preoptic-septal region and were found concentrated along the midline following the curve of the third ventricle from beneath the habenular nucleus and proceeding ventrally through the PVN and the paratenial nucleus. These cells extended ventrally past the third ventricle into the bed nucleus of the stria terminalis, contributing to the increased cell counts in this region. The GnRH content of these cells is approximately five times lower than that found in other areas of the brain as judged by the intensity of immunocytochemical staining (Fig. 3, B compared to C) and the grain densities after in situ hybridization (Fig. 3, D compared to E). These results demonstrate that sequence elements present on the transgene can direct GnRH synthesis in all the normal neuronal sites of GnRH expression as well as the thalamic PVN with high specificity.

Phenotype of rescued mice demonstrates regulated GnRH gene expression. Our results demonstrate that the phenotypic effects of the *hpg* mutation can be reversed by the germ-line integration of an intact mouse GnRH-GAP gene contained on a 13.5-kb DNA fragment. Transgenic animals homozygous for the *hpg* allele developed fully their reproductive functions. Females had estrous cycles and could conceive and carry healthy litters to term. Males displayed correct mating behavior. This phenotypic reversal is due to the neural-specific as well as developmentally regulated expression of the GnRH-GAP precursor gene and the subsequent restoration of the hypothalamic-pituitary-gonadal axis. It is important to note that the hypothalamic neurons expressing the transgene must be correctly controlled in pulsatility and amplitude of GnRH release. This control is believed to occur through a complex set of neural connections with the GnRH-secreting cells (6). These connections are probably not affected by the gene deletion and therefore are intact in the *hpg* mouse or, conversely, are established as a result of embryonal GnRH expression. The importance of this control is illustrated by the failure of preoptic area (POA) brain grafts in the adult *hpg* brains to restore normal reproductive functions in the mutant mouse (13, 14). Thus grafted *hpg* females are in a constant estrous state and exhibit reflux ovulation (13), and grafted males develop near normal-sized testes but display no mating behavior (14). The inability of grafted *hpg* females to maintain estrous cycles indicates that GnRH expression is not being correctly regulated. The failure of these males to mate can be traced to a lack of testosterone in the neonatal *hpg* males, the presence of which is required for adult mating behavior to ensue (15). The development of normal-sized testes was never achieved in grafted males (14) but occurred in transgenic males, indicating either a requirement for pre- or neonatal GnRH expression for the presence of testicular GnRH or GAP (or both).

As expected from the phenotype of the rescued mice, immunocytochemical and in situ hybridization studies on the brains of transgenic animals revealed a normal number of GnRH-containing neurons in every brain area previously reported to be a site of GnRH expression (16). We have shown (3) that *hpg* brains contain no detectable GnRH by immunocytochemistry but that distinct neu-rons in the hypothalamus express an aberrant GnRH mRNA at a low level. Our results showing a good overlap of cells positive for GnRH expression by in situ hybridization and immunocytochemistry indicate that the transgene is being expressed in the same neurons that also express the aberrant *hpg* GnRH mRNA. In addition, a discrete population of neurons in the PVN of the thalamus, which have never been observed to express GnRH, exhibited a low level of GnRH expression. Such uncharacteristic expression was also observed at a low level in the livers of rescued animals. The finding of GnRH expression in cells in PVN of the thalamus and in the liver could be explained by the absence of a negative regulatory element in the transgene. Alternatively, the high transgene copy number could be titrating out a negative repressor present in these cell types. Neither of these sites of expression should influence GnRH concentrations in pituitary portal blood. Cells from the PVN of thalamus do not extend to the median eminence, and any GnRH released from the liver is expected to be rapidly degraded by GnRH-degrading enzymes present in peripheral plasma (17).

The major finding of our study is that the sequences present on the 13.5-kb GnRH genomic fragment are sufficient to direct quantitative GnRH and GAP expression in specific neural areas as well as in the testes and ovaries. Such tissue-specific expression has been reported for several other genes (18), but our results provide, in addition, an example of neural-specific expression in a transgenic animal.

The use of gene replacement to rescue the *hpg* mutation illustrates well the potential uses of gene therapy. Particularly striking is the ability to obtain qualitative and quantitative neural-specific expression and the consequent restoration of the normal circulating levels of the gonadotropins and prolactin. The ability to cure genetic defects by gene replacement has been reported in other systems. A murine β-thalassemia was reversed by the introduction of the natural human β-globin gene (19). Attempts to correct a growth deficiency in the dwarf little (*lit*) mouse, by the introduction of the human and rat growth hormone genes failed to reverse this defect. The use of a heterologous gene fusion (metallothionein–rat growth gene) did restore growth to the dwarf mice, but these mice had vastly elevated growth hormone levels, and the females were sterile (20).

The approach that we have described above can now be extended and used to define the individual roles of GnRH and GAP in regulating gonadotropin release and the role of GAP in modulating prolactin secretion. This neuroendocrinological issue can be further pursued by studying transgenic *hpg* animals containing a mutated GnRH and intact GAP coding sequence and vice versa.

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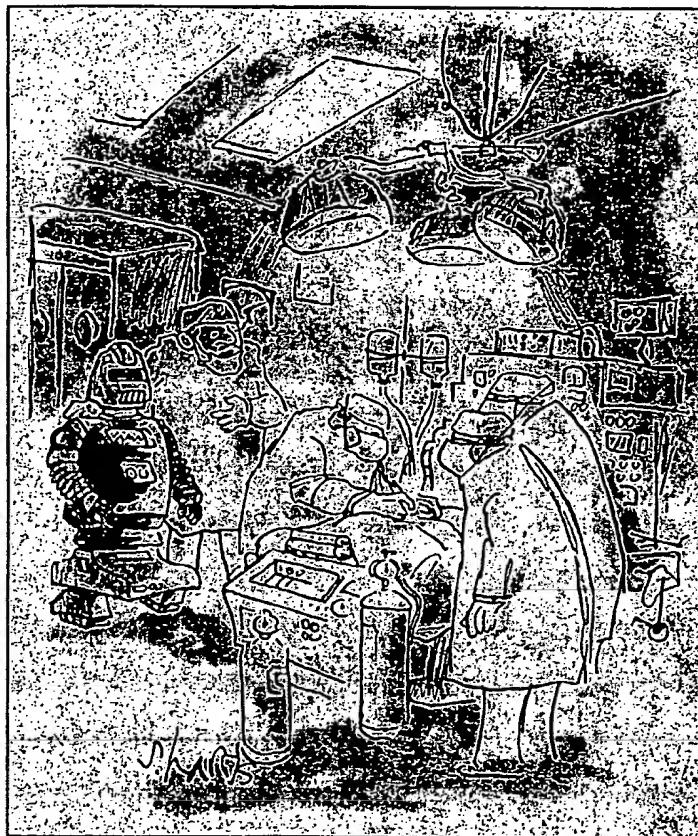
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The Pig as Organ Donor for Man

M. Niekrasz, Y. Ye, L.L. Rolf, N. Zuhdi, and D.K.C. Cooper

THE pig requires little space to breed and be raised, is easy to feed, the costs of breeding and of maintenance are relatively low, it produces large litters, and therefore large numbers are available for organ donation. It grows rapidly, offering organs suitable for even the largest of adult humans. Considerable similarities exist between certain pig organs and those of man, both anatomically and physiologically, namely, size, dietary habits, digestive physiology, kidney structure and function, respiratory rate, pulmonary physiology, coronary artery distribution and hemodynamics, propensity to obesity, susceptibility to disease, and social behavior.

The following facts would be of further advantage—the pig is suitable for gnotobiotic rearing and adapts well to isolation conditions. Ninety million pigs are slaughtered each year in the United States for human consumption, which should minimize criticism.

IMMUNOLOGIC ASPECTS

1. It is necessary to overcome humoral (vascular) hyperacute rejection by removal of preformed antipig antibodies from human plasma prior to transplantation or the "neutralization" of such antibodies at the time of transplantation. Plasma exchange or extracorporeal immunoadsorption, as well as antibody neutralization, are under investigation.
2. Prevention of the further production of antibodies might be achieved by immunosuppression with the currently available drugs or newer agents such as 15-deoxyspergualin, possibly in combination with antithymocyte globulin or pretransplant total lymphoid irradiation.
3. Acute cellular rejection must be overcome using a combination of cyclosporine, azathioprine, and methylprednisolone.
4. Blood group incompatibility does not seem to be a significant factor, according to available information with regard to pig blood groups.

NONIMMUNOLOGICAL ASPECTS

There must be assurance that the pig organ transplanted is anatomically and physiologically normal and that no disease (infectious or neoplastic) is transferred to the human recipient. Transferrable infectious agents and parasites (helminths and protozoa) could best be excluded by the use of gnotobiotic animals. It is not known whether pig organs will function satisfactorily in the human metabolic "milieu."

Disease Processes Affecting Major Organs

The pig heart will be used as an example.[1] Congenital abnormalities include nearly all the anomalies commonly

seen in man. They appear in only 0.16% to 0.49% of pigs (although in one small study the incidence was over 4%). The most common are dysplasia of the tricuspid valve, atrial and ventricular septal defects, and subaortic stenosis.

Other cardiac conditions are (1) pericarditis and bacterial endocarditis (associated with infectious agents), (2) other endocardial lesions, (3) hypertrophic and congestive cardiomyopathy, (4) myocarditis associated with various microorganisms, (5) selenium-vitamin E deficiency cardiomyopathy, (6) myocardial necrosis (in porcine stress syndrome and malignant hyperthermia), and (7) myocardial injury by various toxins. Rhabdomyomatosis and atherosclerosis can be encountered, and migrating nematodal larvae can also cause myocardial damage.

Infectious Agents with Zoonotic Potential

The pig can harbor bacterial, viral, fungal, protozoal, and/or helminth organisms.[2] Breeding and raising pigs in a germ-free environment[3] appears to be the best available solution to the above. Bacterial diseases include erysipelas. Viral infections include influenza, and fungal infections include coccidioidomycosis. Other bacterial, viral, and fungal agents are relatively uncommon. Ectoparasites such as *Sarcoptes scabiei* should prove a negligible problem. Endoparasites such as *Ascaris suum* may be a particularly difficult helminth to exclude. *Toxoplasma gondii* could also prove to be problematic.

Neoplasms

The incidence in the United States is low at 0.004%,[4] although the actual incidence would be higher if pigs were allowed to survive into middle life. (Over 90% of pigs are slaughtered between 4.5 and 6 months of age.) Common neoplasms include skin melanoma, malignant lymphoma, rhabdomyoma of the heart, and embryonal nephroma of the urinary system. Malignant lymphoma is possibly (with embryonal nephroma) the most common tumor found in swine, contributing 46% of all tumors in one survey. It is manifested by pronounced enlargement of lymph nodes. Embryonal nephroma is relatively common in pigs under 1

year of age, but metastases are uncommon. Melanoma, believed to be metastatic from skin primaries, have been found in many internal organs, including the heart. Cardiac rhabdomyoma are generally nonmalignant.

Metabolic Compatibility Between Pig and Man

Will the environmental conditions of the human recipient be satisfactory for good function of grafted organs such as the liver and kidney? Will the necessary metabolites be present to allow normal function? Could minor differences, such as pH or serum hormone levels, have unfavorable effects on graft function? It seems unlikely that every enzyme and every factor will have the same structure in both species.

DISCUSSION

If the immunologic problems can be overcome, the pig might prove a satisfactory organ donor for man. Gnotobiotic rearing would probably be required to ensure that

infectious agents were not transferred. Careful inspection of the pig organ for transplantation could exclude both congenital abnormalities and neoplastic conditions. The pig would provide, however, an unlimited supply of organs, enabling transplantation to be offered to every suitable recipient on a timely basis.

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MUTATIONS IN BRIEF

Identification of 9 Novel IDS Gene Mutations in 19 Unrelated Hunter Syndrome (Mucopolysaccharidosis Type II) Patients; Stanislav L. Karsten,[1]* Elena Voskoboeva,[2] Britt-Marie Carlberg,[1] Wim J. Kleijer,[3] T. Tönnesen,[4] Ulf Pettersson,[1] and Marie-Louise Bondeson[1]; [1]Beijer Laboratory, Department of Genetics and Pathology, Unit of Medical Genetics, Uppsala University, Box 589, S-751 23 Uppsala, Sweden; [2]Institute of Medical Genetics, Russian Academy of Medical Sciences, 115478 Moscow, Russia; [3]Department of Clinical Genetics, University Hospital, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; [4]Department of Biochemistry and Molecular Genetics, The John F. Kennedy Institute, 7 Gl. Landevej, DK-2600 Glostrup, Denmark.

ABSTRACT

Hunter syndrome is an X-linked lysosomal storage disorder caused by a deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS). The IDS deficiency can be caused by several different types of mutations in the IDS gene. We have performed a molecular and mutation analysis of a total of 19 unrelated MPS II patients of different ethnic origin and identified 19 different IDS mutations, 9 of which were novel and unique. SSCP analysis followed by DNA sequencing revealed four novel missense mutations: S143F, associated with the 562C→T polymorphism, C184W, D269V and Y348H. Two novel nonsense mutations were found: Y103X (433C→A) and Y234X (826C→G). In two patients two novel minor insertions (421insA and 499insA) were identified. In one patient a complete IDS deletion was found, extending from locus DXS1185 to locus DXS466. © 1998 Wiley-Liss, Inc.

Hereditary Nonpolyposis Colorectal Cancer: Identification of Novel Germline Mutations in Two Kindreds Not Fulfilling the Amsterdam Criteria; Barbara Quaresima,[1] Cristina Grandinetti,[1] Francesco Baudi,[1] PierFrancesco Tassone,[1] Vito Barbieri,[1] Serafino Conforti,[2] Enrico V. Avvedimento,[1] Francesco Costanzo,[1]* and Salvatore Venuta[1]; [1]Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi di Catanzaro "Magna Græcia", via T. Campanella 115, 88100 Catanzaro, Italy; [2]Unità di Oncologia, Azienda Ospedale di Cosenza, Italy.

ABSTRACT

Hereditary nonpolyposis colon cancer results from heritable defects in the MLH1, MSH2, PMS1 and PMS2 genes, which encode proteins involved in the mismatch repair process. In this work we report the identification of two novel germline mutations in the MLH1 gene from two unrelated HNPCC families. The two affected families do not fulfill the Amsterdam criteria. In family 1 we found a missense S93G mutation, which lies in a MLH1 domain critical for its MMR functions. In family 2 we found a two nucleotide insertion (AG) in position 523 from the AUG which determines an early stop codon at position 606 (codon 203). In both families the mutant alleles cosegregate with the cancer phenotype. © 1998 Wiley-Liss, Inc.

Mutations of the Human Tyrosinase Gene Associated With Tyrosinase Related Oculocutaneous Albinism (OCA1); W.S. Oetting,* J.P. Fryer, and R.A. King; Department of Medicine, University of Minnesota, Minneapolis, MN 55455, USA

ABSTRACT

Mutations in the human tyrosinase gene produce tyrosinase-related oculocutaneous albinism (OCA1, MIM #203100). Tyrosinase is a copper containing enzyme and is responsible for catalyzing the rate limiting step in melanin biosynthesis, the hydroxylation of tyrosine to dopaquinone. We report 13 new mutations in the tyrosinase gene associated with OCA1A (without pigment) and OCA1B (with pigment) including 9 missense mutations (H19Q, R52I, R77C, G97R, C289R, L312V, P313R, F340L and H404P), two nonsense mutations (W80X and R116X) and two frameshift mutations (53delG and 223delG). Our previous work has defined clusters of missense mutations that appear to represent functional domains of the enzyme, and three of the missense mutations fall into these clusters including two (F340L and H404P) that flank the copper B binding site and the missense mutation R52I that is located in the amino terminal end cluster of the protein. The G97R missense mutation is the first identified within the epidermal growth factor (EGF)-like sequence and the H19Q missense mutation alters the cleavage site of the signal peptide sequence. Mutational analysis can provide a definitive diagnosis of the type of OCA as well as help structure/function analysis. © 1998 Wiley-Liss, Inc.

Mutations of the Human P Gene Associated With Type II Oculocutaneous Albinism (OCA2); W.S. Oetting,[1*] J.M. Gardner,[2] J.P. Fryer,[1] A. Ching,[3] D. Durham-Pierre,[4] R.A. King,[1] and M.H. Brilliant[2]; [1]Department of Medicine, University of Minnesota, Minneapolis, MN 55455, USA; [2]Department of Pediatrics, University of Arizona School of Medicine, Tucson, AZ, 85724, USA; [3]E.I. DuPont de Nemouris and Co., Newark, DE 19714, USA; [4]Winston-Salem State University, Winston-Salem, NC 27110, USA
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Grant sponsor: N.I.H.; Grant number: GM22167.

ABSTRACT

Mutations in the human P gene lead to oculocutaneous albinism type 2 (OCA2, MIM #203200), the most common type of albinism in humans. The P gene encodes a 110 kDa protein that is associated with melanosomal membranes and contains 12 potential membrane spanning domains. The specific function of the P protein is currently unknown. We report 7 new mutations in the P gene associated with OCA2. This includes 6 missense mutations (S86R, C112F, A368V, T592I, A724P and A787V) and one frameshift mutation (1047del7). We also report 8 polymorphisms including one amino acid substitution, D/A257. We and others have found many polymorphisms of the P gene in the coding region, several of which result in amino acid substitutions, making molecular diagnosis problematic. In contrast to this is the tyrosinase gene associated with OCA1, with a limited number of polymorphic variations in the coding region. There is also no apparent clustering of P gene missense mutations in contrast to the clustering observed by the tyrosinase gene missense mutations that define functional domains of the protein. Further mutational analysis is needed to help define the critical functional domains of the P protein and to allow a definitive diagnosis of OCA2. © 1998 Wiley-Liss, Inc.

Twelve Novel RB1 Gene Mutations in Patients With Hereditary Retinoblastoma; S. Yilmaz, B. Horsthemke, and D.R. Lohmann*; Institut für Humangenetik, Universitätsklinikum Essen, Germany
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ABSTRACT

Hereditary predisposition to retinoblastoma is caused by germline mutations in the RB1 gene. Mutation analysis in this gene is important because knowledge of the causative mutation is often required for accurate risk prediction in relatives. We have performed RB1 gene mutation analysis in 45 patients with hereditary retinoblastoma. Screening by heteroduplex and SSCP analysis resulted in the identification of small mutations in 28 (62%) patients. Recurrent mutations, mostly CpG-transitions, were found in 16 patients. Two patients with isolated bilateral retinoblastoma showed missense mutations, S567L and C712R, which have previously been reported in a patient with bilateral tumors and in a family with low penetrance, respectively. Twelve of the mutations identified here have not been reported to date. These include a novel missense mutation, L662P, which was identified in two bilaterally affected siblings and their mother with unilateral retinoma. © 1998 Wiley-Liss, Inc.

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Retinoic Acid is a Potent Inhibitor of Inducible Pigmentation in Murine and Hamster Melanoma Cell Lines

Seth J. Orlow, M.D., Ph.D., Ashok K. Chakraborty, Ph.D., and John M. Pawelek, Ph.D.
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Melanocyte-stimulating hormone (MSH) induces melanogenesis in Cloudman mouse melanoma cells. The activities of two enzymes in the melanogenesis pathway, tyrosinase and dopachrome conversion factor, are increased as part of the induction process. Trans retinoic acid (RA), at concentrations as low as 0.1 nM, inhibited the induction of tyrosinase, dopachrome conversion factor, and melanogenesis, but had no effect on the basal levels of either enzyme or of cellular melanin content. Half-maximal effects of RA occurred at a concentration of 10 nM; maximal effects were observed at 1 μM. The effects of RA on melanogenesis were independent of its effects on cellular growth since one Cloudman line tested was growth-inhibited by RA and another was growth-stimulated by RA, but the induction of melanogenesis by MSH in both lines was inhibited by RA. Mixing experiments with cell lysates failed to demonstrate the induction of a tyrosinase inhibitor by RA. The effects of RA were not limited to MSH or to Cloudman melanoma cells since RA blocked cholera toxin-inducible melanogenesis in Cloudman cells, as well as the induction of tyrosinase activity by L-tyrosine in Bomirski hamster melanoma cells. The effects of RA were specific to melanogenesis, however, since RA did not interfere with MSH-induced changes in cellular morphology and growth. Thus, RA appears to be a new and potent tool for understanding mechanisms regulating induction of the pigmentary system. *J Invest Dermatol 94: 461–464, 1990*

All-trans-retinoic acid (RA) is reported to inhibit the growth of a variety of melanoma cell lines [1–4], but conflicting data have been published regarding its effects on melanogenesis, ranging from enhancement [5,6] to inhibition [6–8]. Such studies have exclusively addressed basal rather than inducible levels of melanogenesis.

Melanocyte stimulating hormone (MSH), cholera toxin, methylxanthines, and a number of other agents cause increased tyrosinase activity and melanin production as well as changes in growth and morphology of Cloudman S91 murine melanoma cells [9,10]. In at least one hamster melanoma line, L-tyrosine and L-dopa can also induce both tyrosinase activity and the appearance of melanosomal structures [11].

In this report, we show for the first time that RA is a potent inhibitor of the induction of the pigmentary pathway by MSH, cholera toxin, and L-tyrosine, without affecting the growth and morphologic changes induced by these modulators, or the basal levels of tyrosinase and dopachrome conversion factor activities (DCF). RA should, therefore, be a useful new tool for understanding inductive regulation of melanogenesis.

MATERIALS AND METHODS

Cell Culture The murine melanoma lines Cloudman S91 wt and ins^{d-p} and the hamster melanoma line Bomirski AbC1 and conditions for their culture have been described previously [9,11,12]. All were maintained in Ham's F-10 medium containing 10% horse serum. RA was obtained from Sigma Chemicals (St. Louis, MO), dissolved at 10^{-2} M in DMSO, and kept in foil-wrapped containers protected from light. Cells were plated from 12 to 24 h in plain culture medium prior to any experimental additions. At the end of treatment, cells were removed from the substratum by incubation with Joklik's buffer containing ethylenediaminetetraacetic acid (1 mM), counted with a Coulter counter and pelleted by centrifugation.

Tyrosinase and DCF Assay Cell pellets were lysed in sodium phosphate buffer (0.1 M, pH 6.8) containing Triton X-100 (1%), or stored at −70°C and lysed later. Results were the same whether or not cells had been frozen. The radiometric determination of tyrosinase activity and the spectrophotometric assay of DCF activity were performed as previously described [13,14]. In brief, for tyrosinase determinations, 0.1 ml of cell extract was incubated with 0.01 ml containing 1 μCi L-tyrosine [ring-3,5-^{3}H] (NEN/Dupont, Wilmington, DE; specific activity 50 Ci/mmol) and 5 μg L-dopa in sodium phosphate buffer (0.1 M, pH 6.8) containing Triton X-100 (1%) for 20–60 min at 37°C. One ml of activated charcoal (10% w/v) in 0.1 M citric acid was then added and specimens were centrifuged for 10 min at 2000 × g at 4°C. The supernatants were applied to 0.2 ml columns of Dowex 50 equilibrated in 0.1 M citric acid, washed with 0.5 ml 0.1 M citric acid, and the effluent counted by scintillation spectrometry for the formation of ^{3}H$_2$O. DCF was assayed by adding 0.1 ml of cell extract to 0.5 ml of dopachrome (~0.5 mg/ml) [14] and following the disappearance of absorption at 475 nm. Phenylthiourea (1 mM) was included in DCF assays to inhibit endogenous tyrosinase, which can interfere with the assay.
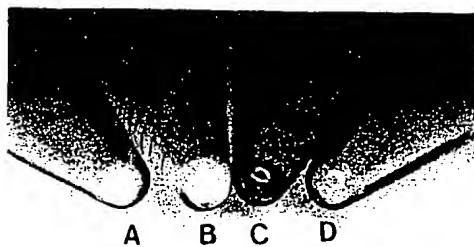
Figure 1. Effects of cholera toxin and RA on cell pigmentation. The cells shown were removed from the substratum with Tyrode's buffer containing 1 mM ethylenediaminetetraacetic acid and pelleted by centrifugation A: no additions; B: RA (1 μM); C: cholera toxin (0.1 nM); D: RA and cholera toxin.

Percent conversion was determined by the following equation:

$$\% \text{ conversion} = \left(\frac{A_{475} \text{ buffer} - A_{475} \text{ sample}}{A_{475} \text{ starting}} \right) \times 100.$$

RESULTS

Cloudman melanoma cells growing in Ham's F-10 medium exhibit a morphology intermediate between polygonal and spindle shaped. Incubation of cells in the presence of cholera toxin (0.1 nM) caused cells to flatten considerably, and to arborize. Inclusion of only trans retinoic acid (1 μM) mildly increased the bipolar spindle phenotype of the cells, but had no apparent morphologic effect on cholera toxin-treated cells (data not shown). Surprisingly, however, retinoic acid had marked effects on the pigmentation of the cells (Fig 1). Cells grown in the presence or absence of retinoic acid alone were amelanotic, but those incubated with cholera toxin alone were heavily melanized. Retinoic acid almost totally inhibited this effect. Pigmentation of cells induced by 200 nM β-MSH was also inhibited by RA (1 μM) (not shown).

These results were reflected in changes in both tyrosinase and dopachrome conversion factor activities. When wild-type Cloudman melanoma cells were grown in Ham's F-10 containing 10% horse serum, little or no tyrosinase activity could be detected (Table I). Incubation of cells for 48 h with β-MSH (200 nM) or cholera toxin (0.1 nM) caused a marked stimulation in tyrosinase activity
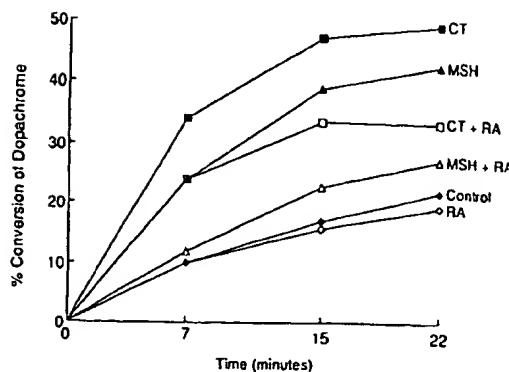
Figure 2. Effects of RA on dopachrome conversion factor activity. Cells were treated for 48 h in the absence or presence of MSH (200 nM) or cholera toxin (0.1 nM) and RA (1 μM). Cell pellets were extracted as described in Materials and Methods, proteins determined, and an aliquot containing 0.1 mg protein assayed in duplicate for DCF as described in Materials and Methods. Shown are the averages of duplicate determinations at each time point which differed by no more than 10%. The experiment was repeated twice with identical results.

(Table I). Inclusion of RA (1 μM) resulted in a 94% inhibition of the MSH-induced stimulation of tyrosinase, and a 77% inhibition of that due to cholera toxin (Table I). These differences were highly statistically significant (p < 0.001). The conversion of dopachrome to dihydroxyindole-2-carboxylic acid, which is catalyzed by DCF, was also stimulated by MSH and cholera toxin ([15] and Fig 2). RA (1 μM) inhibited the DCF stimulation, without affecting basal DCF activity (Fig 2).

The inhibition of tyrosinase stimulation by β-MSH was dose dependent. Effects were detectable at RA concentrations as low as 0.1 nM. Half-maximal inhibition was seen at approximately 10 nM and maximal inhibition was seen at 1 μM (Fig 3). Similar results were observed with the ins^{4-P} Cloudman melanoma line (Table I, bottom). RA was also able to inhibit the stimulation of tyrosinase by isobutylmethylxanthine (100 μM).

The results in Fig 4 demonstrate that if the interval between the time of addition of β-MSH and RA was varied, the inhibitory effects of RA on the stimulation of tyrosinase and DCF activities were diminished. However, even when added 8 h after the MSH, 57% of the maximal inhibition of induction of tyrosinase activity, and 33% of the inhibition of DCF, could still be seen at 1 μM RA. While

Table I. Effect of Retinoic Acid

Treatment[a]	Color of Pellet	Tyrosinase Activity[b]
Induction of pigmentation and tyrosinase activity in wild-type cells		
Control (0.01% DMSO)	white	0 ± 0
RA (1 μM)	white	0 ± 0 NS[c]
MSH (200 nM)	gray	6,108 ± 302
MSH (200 nM) + RA (1 μM)	white	401 ± 76 p < 0.001
CT (0.1 nM)	gray-black	17,115 ± 694
CT (0.1 nM) + RA (1 μM)	light-gray	4,070 ± 183 p < 0.001
Induction of tyrosinase activity in ins^{4-P} cells		
Control (0.01% DMSO)	white	221[d]
RA (10 μM)	white	274
MSH (200 nM)	tan	2,611
IBMX (100 μM)	white	605
MSH (200 nM) + IBMX (100 μM)	dark gray	11,285
MSH (200 nM) + IBMX (100 μM) + RA (10 μM)	white	1,608

[a] Cells were treated for 48 h.
[b] cpm ³H₂O released/5 × 10⁵ cells/h.
[c] Numbers shown are the averages of triplicate determinations ± SD.
[d] Numbers shown are the averages of duplicate determinations which differed by less than 15%.
[e] NS = difference not statistically significant by Student paired t test.

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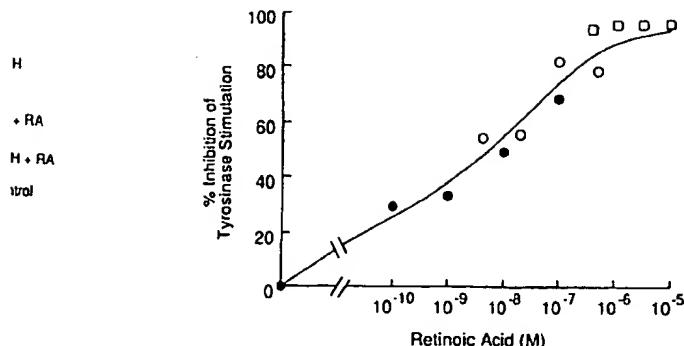
Figure 3. Dose dependence of the inhibition of tyrosinase stimulation by RA. Cells were treated for 48 h with MSH (200 nM) and the indicated concentrations of RA. Tyrosinase activity was assayed as described in *Materials and Methods*. Points represent the averages of triplicate determinations, which differed by less than 15%. Results from three separate experiments spanning the range from 10^{-10} to 10^{-5} M are shown. The *curve* was generated by an Apple statistical best-fit program. The different symbols represent the three overlapping ranges tested (●, 10^{-10}, to 10^{-7} M; O, 5×10^{-9} to 10^{-6} M; □, 5×10^{-7} to 10^{-5} M).

Figure 4. Time dependence of RA effects. Cells were treated with or without MSH (200 nM) for 48 h. RA (1 μM) was added either at the same time or the indicated number of hours later. Tyrosinase and DCF activities were assayed as described in *Materials and Methods*.

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retinoic acid is growth inhibitory to the wild-type Cloudman melanoma line, it is actually growth stimulatory in concentrations up to 3.3 μM to the ins*** line (Table II). The differences between control cultures and those treated with RA were significantly different at all concentrations shown (p < 0.001). Therefore, because RA is inhibitory for induced melanogenesis in both cell lines, such effects are apparently independent of its effects on cellular growth.

The RA inhibition of induced melanogenesis was not specific to β-MSH or cholera toxin, nor a peculiarity of the Cloudman melanoma line. Treatment of Bomirski AbC1 hamster melanoma cells for 48 h with MSH resulted in a fivefold increase in tyrosinase activity (Table III). Also, incubation of the Bomirski cells with 100 μM L-tyrosine supplemented to the medium caused a sixteenfold stimulation in tyrosinase activity. RA (1 μM) caused a 60% and 73% reduction, respectively, in these stimulations. These results were highly statistically significant (p < 0.001).

Mixing experiments were performed to determine whether RA might stimulate the production of a tyrosinase inhibitor. Aliquots from treated and untreated cells were mixed in the indicated proportions and tyrosinase determined. When the values expected on the basis of the arithmetic average of the volumes of the individual samples assayed were compared with the actual values obtained experimentally, no difference was seen, suggesting that no tyrosinase inhibitor was present (Table III). For example, a comparison of lines 7 and 8 in Table III shows that inclusion of extract from a RA-treated cell did not result in any statistically significant diminution in tyrosinase activity.
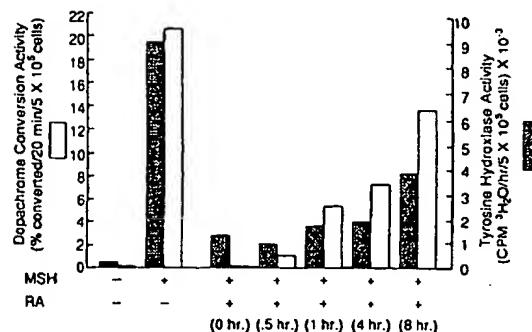
DISCUSSION

The results demonstrate that all-trans-retinoic acid is a potent inhibitor of the induction of two enzyme activities involved in the melanogenesis pathway, tyrosinase and DCF, as well as new melanin-formation elicited by MSH, cholera toxin, or IBMX. The effects of RA on the induction of melanogenesis by MSH and cholera toxin do not extend to changes in morphology and growth caused by these two agents. Half-maximal effects of RA on induced melanogenesis were obtained at concentrations of 10 nM. Although most of our studies were with Cloudman mouse melanoma cells, similar results were obtained with the induction of tyrosinase activity and melanin formation by MSH and L-tyrosine in Bomirski hamster melanoma cells.

It has recently been shown that RA receptors appear to modulate transcription, akin to the action of steroid receptors [16]. It is thus tempting to speculate that tyrosinase and dopachrome conversion factor are in some fashion transcriptionally regulated by RA. However, because only a fraction of the elevated tyrosinase activity in response to MSH seems to be regulated at the transcriptional level [17,18], other models for the action of RA in preventing induced melanogenesis need consideration. RA should prove to be a useful tool for a number of studies on the regulation of melanogenesis. Its specificity for the reversal of the pigmentary effects of MSH and cholera toxin without reversing their growth inhibitory effects may shed light on the transduction of the signal for stimulation of pigmentation in response to such stimuli.

The formation of melanin occurs in melanosomes, which are believed to be the product of the fusion of coated vesicles containing the enzymes responsible for melanization with organelles containing melanosomal structural proteins (see [19] for review). Studies are underway to determine whether RA inhibits only the induction of tyrosinase and DCF activities, or whether the structural protein

Table II. Effect of RA on Growth of Cloudman Melanoma Lines

Treatment*	Cloudman S91 Wild-Type		Cloudman ins***	
	Number of Cells* (× 10³)	% Control	Number of Cells (× 10³)	% Control
None (DMSO 0.01%)	4.8 ± 0.06	100	4.8 ± 0.01	100
RA 3.8 × 10⁻⁷ M	3.5 ± 0.01	73	7.2 ± 0.02	150
RA 1.1 × 10⁻⁶ M	2.9 ± 0.01	61	7.0 ± 0.02	146
RA 3.3 × 10⁻⁶ M	2.8 ± 0.01	58	5.5 ± 0.01	115
RA 1.0 × 10⁻⁵ M	2.6 ± 0.01	50	4.6 ± 0.01	96

*Cells were treated for 72 h.
*Triplicate cultures were counted and averaged. The p value for all samples compared to untreated control (DMSO 0.01%) is p < 0.001.

Table III.   Inhibition of Tyrosinase Activity Induction in Bomirski Hamster Melanoma

Treatment[a]	Tyrosinase Activity[b]	(Expected)	Cell Pellet Color
1. 0 (0.01% DMSO) (100μl)[d]	747 ± 39 \} N.S.[e]		white
2. RA (1 μM) (100μl)	744 ± 177		white
3. MSH (200 nM) (100μl)	3631 ± 73 \} P < 0.001		white
4. MSH (200 nM + RA (1 μM) (100μl)	1883 ± 67		white
5. L-tyr (100 μM) (100μl)	12036 ± 863 \} P < 0.001		gray-black
6. L-tyr (100 μM) + RA (1 μM) (100μl)	3864 ± 601		white
Mixing experiments			
7. 25 μl #5 + 75 μl #1	3725 ± 745 \} N.S.	(3569)	
8. 25 μl #5 + 75 μl #2	4143 ± 144	(3567)	
9. 25 μl #5 + 75 μl #6	6401 ± 350	(5907)	
10. 25 μl #5 + 75 μl buffer	3402 ± 317	(3009)	

[a] Cells were treated for 48 h.
[b] cpm ³H₂O/2.5 × 10⁵ cells/h.
[c] Averages of triplicate determinations ± SD.
[d] Volume of cell extract assayed.
[e] Difference not statistically significant.

pathway and the formation of mature melanosomes is also affected by this retinoid.

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THE PIGMENTARY SYSTEM

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THE

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tical company for each drug they plan to administer.

FRONTISPIECE A: These two individuals illustrate the wide range of skin and hair color [Marianne
Greenwood (right) graciously shared the photographs (from her book Varför Gråter Puman?) that com-
pose this frontispiece.] B: Classical Celtic woman with blue eyes and red hair. C: Typical Scandinavian
with blue eyes. D: Native American. E: A Peruvian girl. F: Himalayan woman and child. G: A Venezue-
lan woman. H: Two teenagers from New Guinea. I: Man from the New Hebrides Islands.

CONTENTS

Chapter 6

The Biogenesis of Melanosomes

SETH J. ORLOW

The granular nature of pigment—for example, within hair shafts—was recognized as soon as pigmented tissues were subjected to examination by the light microscope. Concurrently, researchers recognized a key role for the enzyme tyrosinase in the synthesis of melanin. By 1945, Hermann and Boss found that tyrosinase isolated from the ciliary body of the eye was particulate in nature (Hermann & Boss, 1945). Subsequently, Fitzpatrick and colleagues (Fitzpatrick et al., 1950) noted the granular brown nature of newly formed tyrosine-melanin.

The advent of the electron microscope in the 1950s resulted in a veritable explosion of descriptions of the now visible granules within melanocytes and melanoma cells (Barishak et al., 1961; Barnicot & Birbeck, 1958; Birbeck & Barnicot, 1959; Charles & Ingram, 1959; Clark & Hibbs, 1958; Dalton & Felix, 1953, 1956; Drochmans, 1960; Hu, F. & Cardell, 1962, 1964; Hu, F. & Lesney, 1963; Wellings & Siegel, 1959; Wellings et al., 1960; Zelickson & Hartman, 1961). Barnicot and Birbeck noted the resemblance of the melanocyte pigmentary system to those seen in secretory cells (Barnicot & Birbeck, 1958). They also noted an apparent sequential maturation of the melanin granules within the cell. Although early descriptions of melanin granules differed among the many investigators studying a wide variety of tissues, a coherent picture of the melanin granule began to emerge nonetheless. Our understanding of the nature of this granule was advanced substantially by a pioneering series of studies by Seiji and colleagues, who combined electron microscopy with newly developed techniques for subcellular fractionation and biochemical determination (Seiji et al., 1961). They termed the pigment granule the "melanosome," and delineated four stages in its maturation (Birbeck, 1963; Seiji et al., 1961, 1963). The first stage, Stage I, was a spherical vacuole with poorly organized internal structure. Elliptical Stage II melanosomes demonstrated evidence of a well-organized internal structure, or matrix, within the organelle. By Stage III, the regular and periodic deposition of electron-opaque melanin was evident upon this matrix. Stage IV melanosomes were so melanized that the melanin deposited within them obscured all internal structure.

CURRENT CONCEPTS

The Origin of Premelanosomes

A point of origin for premelanosomes could be deduced by electron microscopy. Conclusions as to this cellular point of origin were based on the proximity of premelanosomes to particular intracellular structures as well as the presence of apparent contiguities between premelanosomes and different subcellular

structures upon serial sections examined by electron microscopy. It was generally agreed that premelanosomes appear to arise from smooth membranous structures, and numerous researchers deduced that these saccular structures represented endoplasmic reticulum or even nuclear membrane (which is contiguous with the ER) (Eppig & Dumont, 1972; Hirobe, 1982; Ide, 1972; Jimbow et al., 1979; Maul, 1969; Maul & Brumbaugh, 1971; Stanka, 1971; Stanka et al., 1981). This area was also therefore referred to as the "condensing vacuole." Within these saccules, which were described as anastomosing and budding like a cactus plant (Allegra, 1974), internal matrix condensed from amorphous filamentous material to an organized structure resembling barrel staves when viewed along the long axis of the organelle (Fig. 6–1).

The nature of the internal melanosomal matrix was subjected to examination by a number of investigators (Hearing et al., 1973; Lutzner & Lowrie, 1972; Moyer, 1963). In some views, the matrix appeared to be composed of filaments arranged parallel to or perpendicular to the long axis of the melanosome. However, on cross section, many investigators noted the presence of spiral elements, suggesting that the matrix was not due to the presence of "two dimensional" filaments, but, rather, to the existence of sheets or lamellae rolled upon themselves like a "jelly roll."

A Role for the Golgi Apparatus

A major advance in the analysis of the biogenesis of melanosomes came from the ability to stain tissues and cultured cells histochemically for the DOPA oxidase activity of tyrosinase itself (Mishima, 1994; Novikoff et al., 1968; Stanka, 1970). It was found that upon incubation of fixed tissue sections with the melanin precursor DOPA, an electron-opaque melanin would be deposited. Surprisingly, in addition to deposition within melanosomes, DOPA-melanin deposition was also noted in the area of the cell called GERL (Golgi-endoplasmic reticulum-lysosomes) (Novikoff et al., 1968), now generally referred to as the trans-Golgi network (TGN), as well as in nearby small vesicles (Maul, 1969; Maul & Brumbaugh, 1971) (Fig. 6–2). Immunoelectron microscopy performed to localize tyrosinase protein confirmed the presence of the enzyme in the TGN saccules and in the adjacent vesicles (Yamamoto & Takeuchi, 1981). Although Stage III melanosomes routinely darkened upon DOPA incubation (and Stage IV melanosomes were already fully melanized), only a subset of Stage II organelles showed evidence of melanin deposition with this procedure. This sequential maturation of melanosomes was subsequently confirmed by studying the changes in melanosome structure and content dur-
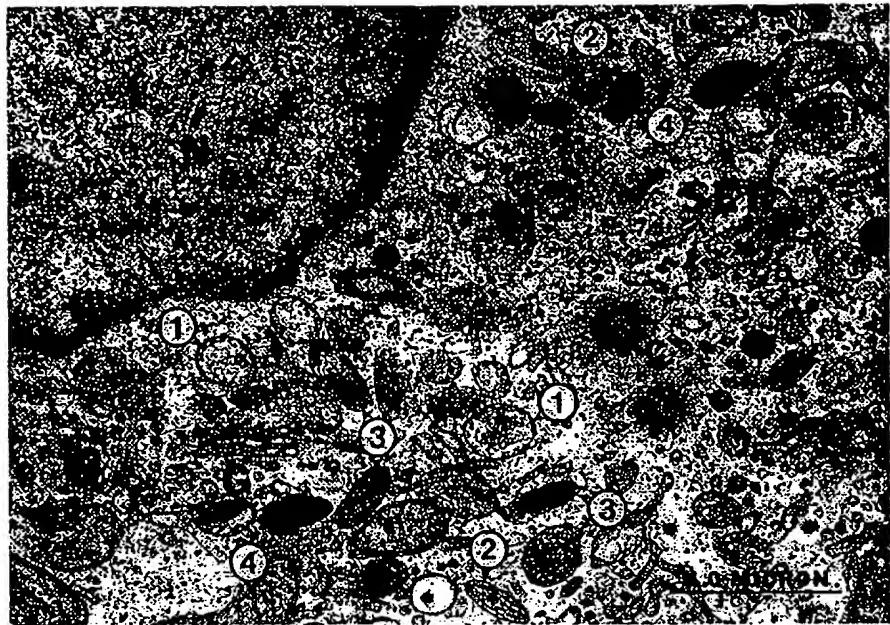
Fig. 6–1. Human melanocyte exhibiting the various stages (1–4) in the development of the melanosome. Stage 1—a relatively spherical organelle with matrix material (i.e., filaments) beginning to assemble; Stage 2—an oval-shaped organelle containing an organized internal matrix without the appearance of melanin; Stage 3—an organelle exhibiting deposition of melanin on the matrix; and Stage 4—an organelle completely filled with melanin. N, nucleus; SER, smooth endoplasmic reticulum; G, Golgi apparatus; C, centriole. Bar, 1.0 μm. [Illustration courtesy of R. E. Boissy.]
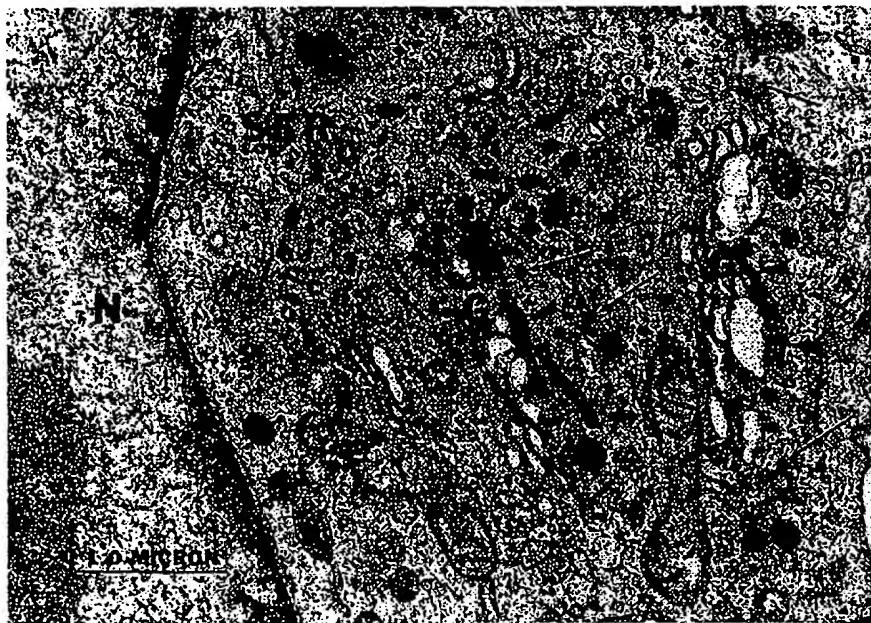
Fig. 6–2. Human melanocyte processed for DOPA histocytochemistry demonstrating electron dense reaction product in the trans most cisternae of the Golgi apparatus (arrowheads), vesicles emigrating from the trans-Golgi network (arrows), and early stage (1–2) melanosomes (open arrows). N, nucleus; SER, smooth endoplasmic reticulum; G, Golgi apparatus. Bar, 1.0 μm. [Illustration courtesy of R. E. Boissy.]

ing developmentally regulated melanogenesis in mice (Hirobe, 1982; Hirobe & Takeuchi, 1977, 1978; Ide, 1972; Quevedo & Fleischmann, 1980).

Thus arose the currently accepted bipartite theory of melanosome biogenesis (Jimbow et al., 1976, 1979). Tyrosinase-charged vesicles, budding off the trans-Golgi network, fuse with smooth-ER-derived premelanosomes (Stage I–II melanosomes), thereby initiating the actual process of melanization. It was hypothesized that it would be advantageous to the melanocyte to restrict melanin deposition to the intramelanosomal compartment, thereby protecting the cell from the deleterious effects of toxic reactive oxygen species generated upon melanization (Hochstein & Cohen, 1963; Riley, 1970).

Mishima and coworkers explored in detail the role of vesicles in melanosome biogenesis (Chakraborty et al., 1989; Mishima & Imokawa, 1983; Mojamdar et al., 1979). They showed that Golgi apparati isolated from melanoma cells could be incubated with DOPA, resulting in the formation of melanin-filled tyrosinase-containing vesicles that could be isolated based on their high density. They also isolated coated vesicles from melanoma cells using techniques developed for the purification of those structures from brain tissue (Chakraborty et al., 1989). They found that the specific activity of tyrosinase in these vesicles was quite high, in keeping with their putative role as specific carriers of the enzyme to the melanosome. Furthermore, they showed that such vesicles contained other enzymes that may be involved in melanization, including glutathione S-transferase, and, in addition, monomeric melanin precursor molecules such as dihydroxyindole-2-carboxylic acid (DHICA) (Chakraborty et al., 1989). Thus, such vesicles contain not only some of the enzymes necessary to synthesize melanin, but appear to contain certain melanogenic intermediates, as well. The fact that, nevertheless, melanin is not actually deposited in these vesicles suggests the presence of additional factors that may prevent true

Fig. 6–3. Murine melanoma cell immunocytochemically process using (A) PEP1 recognizing the carboxy terminus and (B) PEP2 recognizing the amino terminus of TRP-1. The gold-particle associate labeling appears predominantly on the cytoplasmic side using PEP1 (arrowheads in A) and the cisternal side using PEP2 (arrowheads in B) of the limiting membrane of the melanosome. This data is consistent with the prediction that the carboxy end of the putative transmembrane TRP-1 molecule protrudes into the cytoplasm of the cell as the amino end exists within the melanosome proper. Bars, 0.2 μm. [Illustration courtesy of R. E. Boissy.]

melanogenesis until the vesicle contents fuse with a Stage II melanosome.

Vesiculoglobular Bodies

Jimbow and colleagues carefully described the presence within melanosomes of membrane-bounded vesicles of about 40 nm in diameter, which they termed "vesiculoglobular bodies" (Jimbow & Fitzpatrick, 1974; Jimbow & Kukita, 1970). Others had noted such structures previously (Durrer & Villiger, 1967; Tousimis, 1963). These vesicles appeared to be within the melanosome, often between the melanosomal membrane and the matrix, and sometimes apparently associated with the matrix lamellae themselves (Jimbow et al., 1979). The centers of these vesicles failed to melanize, so that their presence in otherwise melanized Stage IV melanosomes lends those organelles the appearance of black "Swiss cheese" on high-resolution examination (Jimbow & Fitzpatrick, 1974).

The evidence seems to support the contention that these vesiculoglobular bodies represent the internalization by inversion of the coated vesicles carrying tyrosinase, a transmembrane protein, following their uncoating at the time of fusion with premelanosomes (Jimbow et al., 1979; Maul & Brumbaugh, 1971; Stanka, 1971; Turner et al., 1975).

The Vesicular Pathway: The TRP Family and the *Silver* Locus Protein

As noted above, it has long been recognized that tyrosinase traffics to melanosomes via coated vesicles. Subsequently, two other members of the tyrosinase gene family, tyrosinase-related protein-1 (TRP-1) (Jackson, 1988) and tyrosinase-related protein-2 (TRP-2) (Jackson et al., 1992), were identified. Subcellular fractionation studies support the presence of both TRP-1 and TRP-2 in the vesicular fraction, as well as in melanosomes (Chakraborty et al., 1989; Donatien & Orlow, 1995). Given the homology between the members of this gene family, that finding was not at all surprising. All three proteins are type 1 membrane proteins, with long intralumenal amino-terminal domains, single transmembrane domains, and short cytosolic carboxyterminal tails. This orientation within the membrane has been formally proven for TRP-1 by immunogold localization at the ultrastructural level (Orlow et al., 1993) (Fig. 6–3). It is believed that these cytosolic tails are critical for proper trafficking of these proteins to melanosomes (see "Signals Directing Melanosomal Trafficking").

A fourth protein that appears to traffic to melanosomes via the same vesicular pathway has also recently been identified and is encoded by the murine *silver* locus (Zhou et al., 1994). It is the antigen recognized by numerous monoclonal antibodies raised against human melanomas, including HMB45, HMB50, NKI/beteb, and ME20 (Adema et al., 1993, 1994; Maresh et al., 1994). This protein is distantly related to the TRP family (Kwon, 1993). Despite its predicted sequence, suggesting that at least one isoform is a type 1 membrane protein, most studies examining the subcellular localization of the murine and human proteins have found it to be associated with the melanosomal internal matrix (Donatien & Orlow, 1995; Zhou et al., 1994). Unlike the TRP family members, at least one form of this protein partitions into the aqueous rather than the detergent phase

A   B   C   D   E

α WM   α TXA   α TXD   αTYR   α PEP13

a si   a si   a si   a si   a si

97 —   97 —   97 —   97 —   97 —

69 —   69 —   69 —   69 —   69 —

46 —   46 —   46 —   46 —   46 —

Fig. 6–4. Organelle scanning. Immunoblotting analysis of wild-type and *si* extracts. Equal quantities of protein (20 µg) from postnuclear supernatants of melan-a (a) and melan-si-1 (si) cells were subjected to SDS/PAGE and immunoblotting with the indicated antisera. *A:* αWM, antiserum to whole melanosomes. *B:* αTXA, antiserum to the aqueous phase of phase-separated, Triton X-114-soluble melanosomal proteins. *C:* αTXD, antiserum to the detergent-soluble phase of phase-separated, Triton X-114-soluble melanosomal proteins. *D:* αTYR, anti-peptide antiserum (αPEP7) to the carboxyl terminus of murine tyrosinase. *E:* αPEP13, anti-peptide antiserum to the predicted carboxyl terminus of the *si* locus gene product. The arrow indicates an 85-kDa antigen absent from melan-si-1 extracts when analyzed by both αTXA and αPEP13 antisera. Numbers at the left indicated the migration of markers of known molecular size (in kDa). [Reproduced from Zhou *et al.*, PNAS (1994), with permission.]

upon phase separation following solubilization with the nonionic detergent Triton X-114 (Zhou *et al.*, 1994) (Fig. 6–4). Furthermore, several researchers have detected soluble secreted forms of this protein in cultured melanoma cells (Maresh *et al.*, 1994). The *silver* locus protein appears to interact closely and specifically with melanin during its deposition; the protein becomes selectively incorporated into the nascent melanin polymer rapidly upon the initiation of melanization *in vivo* and *in vitro* (Donatien & Orlow, 1995).

Nonetheless, since a well-organized matrix is evident prior to fusion of premelanosomes with the vesicles that would contain the *silver* locus protein, it is highly unlikely that this protein represents the basic structural element of the melanosomal matrix. Rather, it might be better envisioned as a nucleation point for the attachment of the developing melanin deposits with the organelle.

The Pink-Eyed Dilution Gene and Melanosome Biogenesis

Fifty years ago, Russell (1949) noted by light microscopy that the pigment granules in the hairs of mice homozygous for the pink-eyed dilution (*p*) mutation were small and shredlike. Some (but not all) investigators in the 1960s and 1970s found abnormalities in the structure of melanosomes in the eyes and skins of *p* mice by electron microscopy (Hearing *et al.*, 1973; Moyer, 1963, 1966; Rittenhouse, 1968). Among these descrip-

tions are ones by Moyer (1963, 1966) and Rittenhouse (1968) which note that the matrix of *p* melanosomes appeared disorganized and that the melanosomes themselves were small. Recently, preliminary results were reported by Boissy and colleagues (Zhao *et al.*, 1995), who examined melanocytes cultured from persons with OCA2 (now known to be due to mutations in the human homolog of the murine *p* gene (Durham-Pierre *et al.*, 1994; Lee *et al.*, 1994; Rinchik *et al.*, 1993; Spritz *et al.*, 1995). They, too, noted an abnormality in melanosome shape, matrix disorganization, and a diminution in the number of melanized melanosomes. Taken together with the finding that melanocytes cultured from *p*-null mice exhibit a marked diminution in melanosome size (Rosemblat *et al.*, 1996), the data support the conclusion that the *p* gene product is critical for proper melanosome biogenesis and structure.

As mentioned above, the currently accepted model for melanosome biogenesis invokes a bipartite pathway, in which premelanosomes containing a lamellar matrix bud from the smooth endoplasmic reticulum and fuse with vesicles derived from the trans-Golgi network and charged with members of the TRP family (Jimbow *et al.*, 1976). It is the fusion of these structural and enzymatic elements that initiates the process of melanogenesis. Previous results from my laboratory (Rosemblat *et al.*, 1994) have demonstrated that the p protein appears to be inserted directly into premelanosomes, bypassing the TGN-derived vesicles. Furthermore, evidence for Asn-linked glycosylation of the p protein could not be detected, also consistent with the behavior of a protein that bypasses the Golgi appara-

tus. If the p protein is indeed a premelanosomal component, one can readily imagine that the absence of this protein might severely compromise the ability of the melanocyte to properly construct a melanosome.

Recent evidence also suggests that the p protein may interact specifically with melanin, becoming progressively more insoluble upon melanin deposition (Donatien & Orlow, 1995). It is certainly possible that the p protein may serve to link the intramelanosomal matrix to the melanosomal membrane, since the two always grow in concert. Diminished levels of tyrosinase activity (Chiu et al., 1993) and protein (Chiu et al., 1993) have been found in p mutant mouse tissues in the past. The p^{un} mutation, which is due to an in-frame tandem duplication affecting the second through the seventh predicted transmembrane domains (Oakey et al., 1996), results in a marked diminution in levels of the TRP family in the ocular tissues of affected neonatal mice homozygous for the mutation (Chiu et al., 1993). Furthermore, the high-molecular-weight forms of TRP family members were also diminished in these mice, perhaps the result of diminished stability (Chiu et al., 1993; Lamoreux et al., 1995). Culture of cells from p-null mutant mice in tyrosine results in increased melanization, as well as increased levels of tyrosinase and the levels of certain melanosomal matrix proteins (Rosemblat et al., 1998). This effect appears to be at the protein level, since levels of mRNA for tyrosinase are the same before and after tyrosine stimulation (Rosemblat et al., 1998). In addition to its effects on melanosomal proteins, tyrosine stimulation of p-null melanocytes upregulates cellular melanization, and melanosome number and structure (Rosemblat et al., 1998). This augmentation of melanization by tyrosine has been taken as supportive of a role for the p protein as a tyrosine transporter. Certainly, the predicted secondary structure of the p protein is in keeping with a channel or transporter (Gardner et al., 1992; Rinchik et al., 1993). It is possible that high intramelanosomal tyrosine levels stabilize the matrix and hence the structure of melanosomes, as well as of tyrosinase when it arrives at its destination via coated vesicles. Nonetheless, these effects of tyrosine are by no means specific to p melanocytes, but are also observed in melanocytes from wild-type black mice, too (Rosemblat et al., 1998). Furthermore, recent results from Gahl and colleagues (1995) demonstrate that although melanosomes appear to possess a specific tyrosine transport system, melanocytes from mice homozygous for a p gene deletion (p^{cp}/p^{cp}) show tyrosine transport activity indistinguishable from black controls in a variety of subcellular compartments, including melanosomes.

While the precise role of the p protein remains to be delineated, it is clear that the absence of the p gene product has serious implications for melanosome number, structure, and melanization, and supports the contention that this protein plays a central role in the biogenesis of the melanosome. Our ability to study these issues in detail should be greatly aided by the in vitro systems now available.

Melanosomal-Lysosomal Relationship

It had long been suspected that melanosomes might be related to lysosomes. By the late 1960s cytochemical techniques could be used to detect the presence of acid phosphatase activity, considered a hallmark of lysosomes, within melanosomes (Novikoff et al., 1968; Seiji & Kikuchi, 1969; Wolff & Schreiner, 1971).

Genetic evidence was subsequently developed in support of a commonality between melanosomes and lysosomes. During the 1970s, a number of single-gene mutations causing pigmentary dilution in the laboratory mouse were found to be associated with decreased secretion of kidney lysosomal hydrolases, especially β-glucuronidase, β-hexosaminidase, and β-galactosidase, into the urine (Hakansson & Lundin, 1977; Meisler et al., 1980; Novak & Swank, 1979). Although tissues from mice homozygous for most of these mutations were devoid of distinctive microscopic changes, mice homozygous for the beige (bg) mutation demonstrated the presence of giant granules, including giant melanosomes, in a variety of tissues (Hearing et al., 1973; Lutzner & Lowrie, 1972; Oliver & Essner, 1973). Electron microscopy and histochemistry revealed that many of these granules represented specialized lysosomes present within the affected tissues (Oliver & Essner, 1973, 1975). These include leukocyte granules, especially the azurophilic granules of the neutrophil and the lytic granules of natural killer cells, as well as melanosomes in melanocytes. Melanosomes were consequently implicated as members of the lysosomal lineage of organelles. The markedly decreased number of these giant lysosomally derived granules suggests that they arise from the uncontrolled fusion of normal-size granules. This supposition is strengthened by direct observation of fibroblasts cultured from beige mice, in which the fusion of lysosomes to form the giant granules has been documented (Willingham et al., 1981).

Concurrently, a number of human genetic diseases affecting both skin and eye color as well as lysosomes and lysosomally derived organelles were also identified. The distinctive microscopic findings in the Chediak-Higashi syndrome (Windhorst et al., 1966; Zelickson et al., 1967) suggested that it was homologous to the beige mutation in mice. Melanocytes cultured from a patient with CHS exhibited aberrant and selective secretion of tyrosinase and β-glucuronidase into the media (Zhao et al., 1994). Persons with Hermansky-Pudlak syndrome (Hermansky & Pudlak, 1959), a form of oculocutaneous albinism in man (Witkop et al., 1989), have a lysosomal defect manifested by the accumulation of a ceroid-lipofuscin material in the lysosomes of macrophages of the lung and gut, leading to a restrictive lung disease (Garay et al., 1979) and granulomatous colitis (Schinella et al., 1980) in many affected persons. Persons with Hermansky-Pudlak syndrome also have a bleeding diathesis caused by the absence of platelet dense granules with a resultant defect in platelet aggregation.

Many of the mutations in the mouse affecting lysosomes also result in a platelet aggregation defect (Novak & Swank, 1979; Novak et al., 1984, 1985), apparently due to the presence of defective platelet dense granules. The platelet dense granule is a subcellular organelle that is also affected by many of the same mutations that affect lysosomes and melanosomes, suggesting that these three organelles are all related (Orlow, 1994, 1995). Platelet dense granules contain serotonin, calcium, and ATP, and are important for the second irreversible wave of platelet aggregation. The membrane of the dense granule contains a 40 kDa protein, called granulophysin (Gerrard et al., 1991; Nishibori et al., 1993), which is also expressed in a granular pattern by melanocytes (Zhao et al., 1994) (Fig.6-4). The number of platelet granules is decreased in beige mice and in persons with Chediak-Higashi syndrome (Costa et al., 1976; Meyers & Seachord, 1990; Novak et al., 1984, 1985).

A hallmark of the endolysosomal lineage of organelles is accessibility to material endocytosed from the extracellular environment. Proteins and hormones internalized via coated pits

pass through endosomes to accumulate in lysosomes. Moell-mann and colleagues demonstrated that ferritin-labeled mela-nocyte-stimulating hormone (MSH) bound to the surface of Cloudman S91 melanoma cells could be internalized and at least a small percentage could be shown to enter melanosomes, suggesting that melanosomes, like lysosomes, are accessible to endosomal traffic (Moellmann *et al.*, 1988). Moellmann and coworkers subsequently showed that binding of MSH to Cloud-man S91 melanoma cells resulted in an acidification of the TGN and melanosomes as assessed by the accumulation of the weak base DAMP within these organelles. A low internal pH of mela-nosomes (4–4.5 or lower) was confirmed by Ramaiah and col-leagues (Bhatnagar *et al.*, 1993) utilizing fluorescent dye mole-cules sensitive to the pH of intracellular organelles. The more highly melanized melanosomes appeared to have a progres-sively lower internal pH (Bhatnagar *et al.*, 1993).

A flurry of recent studies have brought modern techniques of cell biology to bear on the issue of the relationship between me-lanosomes and the endosomal/lysosomal lineage of organelles. It has been recently shown that Stage I–II melanosomes contain not only the expected melanosomal proteins such as tyrosinase and tyrosinase-related protein-1, but also the lysosomal/late en-dosomal marker proteins LAMP-1 and LAMP-2 (Orlow *et al.*, 1993; Smit *et al.*, 1993; Zhou *et al.*, 1993). Concurrently, normal human melanocytes and melanoma cells were shown to be capa-ble of phagocytosing latex beads, and moreover, the phago-somes containing these beads were shown to be capable of fu-sion with melanosomes (Le Poole *et al.*, 1993; Mishima, 1994).

If melanosomes share a common biogenesis with endolysoso-mal organelles, then one would predict that, when expressed in nonmelanocytic cells, melanosomal proteins might localize to lysosomes instead. Evidence from a number of independent lab-oratories lend support to this prediction. Analyses of fibroblasts transfected with vectors directing the expression of tyrosinase or the related protein TRP-1 demonstrate that the exogenously ex-pressed proteins localize to spherical perinuclear granules that contain LAMP-1 (Vijayasaradhi *et al.*, 1991) and lysosomal hy-drolases such as β-glucuronidase (Winder, 1991); these are char-acteristics of both late endosomes and lysosomes (Kornfeld & Mellman, 1989). The colocalization of exogenously expressed melanosomal proteins with the cation-dependent mannose 6-phosphate receptor (which is present in endosomes but absent from lysosomes) has not yet been reported upon.

Taken together, the aforementioned results cannot differenti-ate between the possibility that melanosomes and lysosomes are divergent organelles that share a common biogenesis or, al-ternatively, that melanosomes may actually represent a highly specialized subset of lysosomes. To specifically address this question, we and others have recently examined the presence of classical lysosomal hydrolases within melanosomes. The re-sults suggest that melanosomes are the major repository of lysosomal glycosidases within melanocytic cells, consistent with their role as specialized lysosomes (Diment *et al.*, 1995; Smit *et al.*, 1993).

More surprising is our observation that the absolute levels of a subset of lysosomal hydrolases is markedly increased in highly melanized melanocytes when compared with less mela-nized cells (Diment *et al.*, 1995). Furthermore, nonmelanocytic cells such as fibroblasts transfected with the gene encoding ty-rosinase demonstrate elevated levels of expression of certain lysosomal hydrolases (Borovansky *et al.*, 1997). Thus, cells ap-pear to possess a feedback loop wherein the presence of ongo-ing melanogenesis results in increased levels of expression of a subset of lysosomal hydrolases. Whether this response protects the cells from the potentially deleterious effects of melanogene-sis is not yet known.

Signals Directing Melanosomal Trafficking

How are the proteins destined for melanosomes correctly tar-geted to those organelles? For those type 1 membrane proteins that traffic to melanosomes via the vesicular pathway, recent evidence suggests that the cytosolic carboxy termini contain specific amino acid sequences that direct their proper trafficking.

Vijayasaradhi and coworkers (Vijayasaradhi *et al.*, 1995) have employed molecular techniques to demonstrate that the cytosolic tail of the *brown* locus protein (TRP-1 or gp75) is sufficient to direct its proper trafficking. When this tail is "grafted" onto the extracellular and transmembrane domains of the plasma membrane protein CD8, the hybrid protein is instead routed to melanosomes. Furthermore, if the carboxy terminal tail of TRP-1 is deleted, the mutant protein instead traffics to the plasma membrane. They were able to pinpoint the region of the carboxy terminal tail that is critical to the last 27 amino acids. Furthermore, they presented evidence that a hexapeptide sequence within this tail, QPLLTD, might be the actual se-quence responsible for the melanosomal trafficking.

These findings are strongly supported by the recent work by Beermann *et al.* (1995) on the *platinum* mutation at the tyrosi-nase locus in mouse. Molecular cloning of this mutant allele re-veals that it results in an A→T substitution that causes a change from a lysine to a stop codon. Thus, the mutant protein is trun-cated by a foreshortening of its tail by the last 27 amino acids. The truncated protein still behaves as an integral membrane pro-tein, but by histochemical and electron microscopic techniques, it can be shown to traffic to the plasma membrane of melano-cytes in the developing mouse eye or in cultured melanocytes derived from skins of neonatal platinum mice (Fig. 6–5). Al-though in ocular tissues, positive staining by the DOPA tech-nique was present in the trans-Golgi network and in nearby vesi-cles, no tyrosinase activity was detected in the melanosomes, which, though devoid of melanin, were otherwise normal. Thus, both for tyrosinase and for TRP-1, the 27 carboxy terminal amino acids are critical for proper subcellular trafficking.

It is interesting to note that the sequence QPLLMD is present in both murine and human tyrosinase, and NSPLLG is found in the Pmel 17/silver gene product. Of significance, however, is the absence of this sequence or a homologous one from the tail of TRP-2, which is also a melanosomal protein.

Of note, however, is a Gly-Tyr motif in the carboxy termini of all of these proteins. This is particularly interesting since such a motif is believed critical for the appropriate sorting of a family of lysosomal glycoproteins, including LAMP-1 (Harter & Mell-man, 1992; Matthews *et al.*, 1992; Williams & Fukuda, 1990). It should be kept in mind that LAMP-1 is also found in melano-somes in melanocytic cells (Orlow *et al.*, 1993; Zhou *et al.*, 1993). Furthermore, when the genes encoding them are trans-fected into nonmelanocytic cells such as fibroblasts, TRP-1 and tyrosinase traffic to endolysosomal compartments that contain LAMP-1 and β-glucuronidase, demonstrating that in the ab-sence of authentic melanosomes, the sequences of TRP family members contain the information necessary for trafficking to en-

Fig. 6–5. Melanocytes (M) established in culture from skin of neonatal platinum mice homozygous for the c[p] allele at the tyrosinase loci processed for DOPA histochemistry. Electron-dense reaction product occurred at the plasma membrane of each melanocyte (arrows) and was prominent between two apposing melanocytes (bold arrows and circled inset). In addition, reaction product was relatively absent from melanosomes (circled inset) and small cytoplasmic vesicles (arrowhead in circled inset), some of which appeared to be fusing with the plasma membrane (arrow in circled inset). Bar, 5.0 μm. [Illustration courtesy of R. E. Boissy.]

dosomes/lysosomes. The Gly-Tyr sequence is also present in the carboxy terminal tail of TRP-2 (Jackson et al., 1992).

Although the sequences NTPLLR and TPLLWN are found, respectively, in the amino terminal region of the human and murine pink-eyed dilution locus protein (Gardner et al., 1992; Rinchik et al., 1993), and although this portion of the protein is predicted to be exposed to the cytosol, evidence suggests that the p protein is inserted directly into premelanosomes and does not pass through the Golgi or the Golgi-derived transport vesicles (Rosemblat et al., 1994). Whether this hexapeptide sequence plays any role at all in the trafficking of the p protein must await further studies.

Pheomelanosomes

Pheomelanins, or red/yellow melanins, differ from eumelanins (black/brown) not only in their chemical composition (they are rich in cysteinyl groups) (Hu, D.-N. et al., 1995; Prota, 1972; Prota et al., 1995; Rorsman et al., 1973) but also in the melanosomes in which they are deposited. In mice that produce

pheomelanin due to mutation at the extension (e) or agouti (A) loci (Moyer, 1966; Sakurai et al., 1975), and in pheomelanic chicks (Jimbow et al., 1979), these "pheomelanosomes" are spherical and devoid of the well-organized internal filamentous or lamellar structure of their eumelanic counterparts. They are, however, rich in internal vesicles.

It is now clear that the levels of a number of key melanosomal proteins are markedly diminished under conditions of pheomelanogenesis. Transcripts for the p-locus protein, which is critical to the normal biogenesis of eumelanosomes (see above, under "The Pink-Eyed Dilution Gene and Melanosome Biogenesis") are absent from the skins of yellow mice (Gardner et al., 1992; Rinchik et al., 1993), as are the protein products TRP-1 and TRP-2 (Lamoreux et al., 1995). Levels of tyrosinase are reduced to about 30% of normal (Lamoreux et al., 1995). Also absent is the silver locus protein (Kobayashi et al., 1995). Thus, one may hypothesize that the pheomelanosome is a "primitive" melanosome containing tyrosinase but lacking other key melanosomal components. In support of this hypothesis is the observation that fibroblasts transfected with the tyrosinase gene make low levels of melanin that chemically resembles pheomelanin (Winder et al., 1993). Of course, in such cells, all other melanosomal components are lacking. The true nature of pheomelanosomes could not previously be studied in vitro since melanocytes from yellow mice produce black eumelanin in vitro. However, now that recombinant agouti protein is available, it can be added to cultured melanocytes and its effects studied. Preliminary evidence from Hearing and colleagues suggests that the content of melanosomal protein as well as melanosomal structure are indeed altered by such in vitro treatment (Kobayashi et al., 1995).

PERSPECTIVES

The process of melanogenesis involves the production of active oxygen species that are toxic to the cell (Borovansky et al., 1991; Hochstein & Cohen, 1963; Pawelek & Lerner, 1978). It may be supposed that the complex structure of the melanosome evolved in order to sequester this toxic series of reactions within a specialized organelle, apart from the rest of the cell's components. The lysosome appears to offer an ideal basis for building such an organelle, as its membrane is coated with highly glycosylated proteins, and the progressive internal acidification of the endolysosomal lineage of organelles allows the cell to tightly control the activity of the many hydrolases contained therein. Indeed, it is recognized that the pH is critical for appropriate melanogenesis, and that the polymerization of melanin is altered in vitro by changes in pH. Thus, the melanocyte may have adapted this basic structure to meet its own specialized needs over the course of evolution.

Summary

1. Melanin is synthesized and deposited within a specialized organelle termed the melanosome. Our knowledge of the structure of melanosomes, how they are constructed by the melanocyte, and how their components are directed to them is the subject of this chapter.

2. In the bipartite theory of melanosome biogenesis, the premelanosome originates from the endoplasmic reticulum and eventually fuses with Golgi-derived vesicles containing the enzymes (i.e., tyrosinase, TRP-1, and TRP-2) involved in melanin synthesis.

3. The p protein (product of the murine pink-eyed dilution gene) is essential for the normal development of melanosome number, structure and melanization. However, its function in this manner is unknown.

4. Melanosomes and lysosomes exhibit common properties, including (*1*) initial biogenesis, (2) LAMP expression, (*3*) presence of hydrolases, and (*4*) genetic syndromes affecting both organelles.

5. The trafficking of molecules to the melanosome is beginning to be resolved. Amino acid motifs in the carboxy termini of TRP-1 and tyrosinase appear crucial for correct targeting.

6. Pheomelanosomes appear to be a primitive melanosome-containing tyrosinase but lack other key melanosomal components.

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Elastase I Promoter Directs Expression of Human Growth Hormone and SV40 T Antigen Genes to Pancreatic Acinar Cells in Transgenic Mice

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The exocrine pancreas, which differentiates from the endothelial cells of the foregut, functions to synthesize and store digestive enzymes that are released into the intestinal tract. The pancreas also contains endocrine cells probably of ectodermal origin, which produce metabolic regulatory hormones, including insulin, glucagon, and somatostatin. During mouse development the pancreatic acinar cells can be detected by about day 10 and begin to synthesize low levels of digestive enzymes between days 11 and 14. Shortly before birth the levels of these enzymes increase dramatically and adult levels are attained within the first several weeks of life (Rutter et al. 1972). Expression of pancreatic serine protease genes is several orders of magnitude higher in the acinar cell than in any other cell type (Swift et al. 1984a). Because expression of these digestive enzymes in inappropriate tissues could be deleterious to an organism, there may be strong selective pressure to maintain this very precise cell-specific expression. To gain an understanding of the regulatory mechanisms governing this family of digestive enzymes, we have begun studying the regulation of the rat elastase I gene. This gene is one of at least nine serine protease genes expressed exclusively in pancreatic acinar cells.

Regulation of cell-specific gene expression is being studied by introducing various genes into tissue culture cells that maintain a differentiated phenotype or by producing transgenic mice carrying these genes. Although cis-acting, cell-specific elements have been identified for a variety of genes by DNA-mediated transfection into cells (Banerji et al. 1983; Chao et al. 1983; Gillies et al. 1983; Kondoh et al. 1983; Stafford and Queen 1983; Walker et al. 1983), transgenic mice have the distinct advantage that developmentally regulated genes can be assayed in every possible cell type throughout the normal development of the mouse. Furthermore, normal levels of expression are observed for many genes in transgenic mice, whereas in tissue culture the level of expression of differentiated genes is often very low. In transgenic mice, tissue-specific expression has been demonstrated for immunoglobulin heavy- and light-chain genes (Brinster et al. 1983; Grosschedl et al. 1984; Storb et al. 1984; Rusconi and Kohler 1985), the elastase I gene (Swift et al. 1984a;
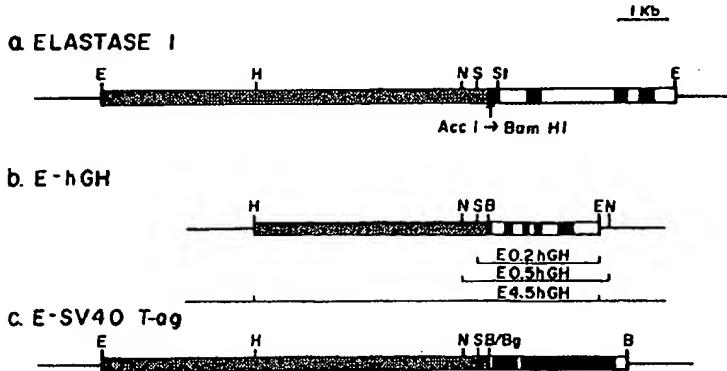
Figure 1. Elastase-hGH and elastase-SV40 fusion gene constructions. (a) A portion of the rat elastase I gene with 7.2 kb of 5' sequence and 3.6 kb of structural gene containing the first four exons. The AccI site at +8 was converted to a BamHI site, as described in Experimental Procedures. (b) The three elastase-hGH fusion genes introduced into transgenic mice. (c) The elastase-SV40 T antigen fusion gene introduced into transgenic mice. Black bars represent exons, stippled regions represent elastase 5'-flanking sequence, and the thin line represents pBR322 vector sequences. (Bg) BglII; (E) EcoRI; (H) HindIII; (N) NdeI; (S) SalI; (St) StuI.

400 ORNITZ ET AL.

Ornitz et al. 1985), the insulin gene (Hanahan 1985), β-globin genes (Chada et al. 1985; Townes et al. 1985), the myosin light-chain gene (Shani 1985), and the α-fetoprotein gene (Krumlauf et al. 1985).

Previous studies in transgenic mice have demonstrated that the entire rat elastase I gene, containing 7 kb of 5' sequence, 11 kb of coding sequence and introns, and 5 kb of 3' flanking sequence, is expressed higher in the pancreas than in any other tissue by three to four orders of magnitude (Swift et al. 1984a). To identify sequences required for pancreas-specific expression in the 23-kb rat elastase I genomic clone, we tested fusion genes containing 5' elastase I sequence and the human growth hormone (hGH) structural gene in transgenic mice (Ornitz et al. 1985). These studies revealed that no more than 213 bp of elastase 5'-flanking sequence are required for acinar cell-specific expression.

The hGH structural gene has several advantages for use in transgenic mice. The gene is small, the five exons span 1.6 kb of genomic DNA (Seeburg 1982, Fig. 1b), and the mRNA appears to be spliced normally and it is reasonably stable in many tissues (Palmiter et al. 1983). The protein product is easily detected by radioimmune assay (Palmiter et al. 1983) or indirect immunofluorescence (Ornitz et al. 1985). Furthermore, if hGH is synthesized in a cell that secretes into the bloodstream, it can stimulate the growth of transgenic mice, a parameter that can be monitored easily (Palmiter et al. 1983). However, if hGH is produced in an exocrine cell type, and consequently secreted externally, then no enhanced growth would be expected.

In this paper we demonstrate that 213 bp of elastase 5' sequence are sufficient for normal levels of pancreas-specific expression, that the tissue-specificity is manifested at the transcriptional level, and that expression of an elastase-hGH fusion gene in transgenic mice correlates with a tissue-specific DNase I hypersensitive site corresponding to the region of the elastase regulatory sequence. We further demonstrate that the elastase regulatory region can direct expression of SV40 T antigen in the pancreatic acinar cells resulting in characteristic pancreatic tumors in transgenic mice.

EXPERIMENTAL PROCEDURES

Construction of fusion genes. As a first step in constructing pancreatic acinar cell-specific expression vectors for use in transgenic mice, we introduced a unique restriction site in the first exon of the rat elastase I gene. To accomplish this, we subcloned a 400-bp *SalI–StuI* fragment that spans the mRNA initiation site and contains 205 bp of elastase 5'-flanking sequence from plasmid Elastase I (containing 7.2 kb of elastase 5' sequence and 3.6 kb of the structural gene; Fig. 1a) into pUC13. A *Bam*HI linker was placed at the *Acc*I site (position +8) in this subclone (E0.2). An elastase-hGH fusion gene (E0.2hGH; Fig. 1b) was made by cloning the hGH structural gene (on a 2.1-kb *Bam*HI-*Eco*RI fragment) into E0.2. Elastase 5'-flanking se-

quence was added to this construction to generate E4.5hGH (Fig. 1b), a plasmid containing 4.5 kb of elastase 5'-flanking sequence. The E7.2 SV40 T antigen fusion gene (Fig. 1c) was made in a similar fashion. The fusion in this construction is at +8 of elastase and at the *Stu*I site at +34 (converted to a *Bgl*II site) in SV40. The resulting mRNA should contain 8 bp of elastase 5' untranslated sequence, a synthetic linker fusion (*Bam*HI/*Bgl*II), and 27 bp of SV40 5' untranslated mRNA, followed by T antigen coding sequence.

Production of transgenic mice. All plasmids were linearized prior to microinjection. E4.5hGH (Fig. 1b) was linearized at the *Eco*RI site prior to injection, E0.2hGH was linearized at *Sal*I, and E7.2SV40 T antigen (Fig. 1c) was linearized at an *Xho*I site in the plasmid vector. The E0.5hGH (a 2.8-kb *Nde*I fragment) and some of the E0.2hGH (a 2.3-kb *Sal*I-*Eco*RI fragment) mice were produced with genes that were separated from vector sequences prior to injection. The restricted fragments were isolated from 1% agarose gels by a perchlorate elution procedure (Chen and Thomas 1980), and 100–300 copies were microinjected into the male pronucleus of fertilized mouse eggs as described (Brinster et al. 1981, 1985). The injected eggs were introduced into the oviduct of pseudopregnant foster mothers and allowed to develop to term. Mouse pups were screened by tail DNA dot hybridization for the presence of the injected gene. Growth rates of elastase-hGH transgenic mice were monitored until they were about 10 weeks old, at which time the mice were either bred to establish transgenic lines or sacrificed for tissue analysis. Elastase SV40 mice were bred and monitored for signs of pathology.

Nucleic acid isolation and RNA analysis. Fifty milligrams of each tissue were homogenized in 4 ml of SET (1% SDS, 1 mM EDTA, 10 mM Tris, pH 7.5) with 100 μg/ml proteinase K. Total nucleic acids were purified as previously described (Durnam and Palmiter 1983) and stored frozen in 0.2 × SET. mRNA levels were analyzed by a solution hybridization procedure utilizing a complementary end-labeled 21-base oligonucleotide probe. Hybridization and S1 nuclease digestion were carried out as previously described (Ornitz et al. 1985).

Isolation of nuclei. Nuclei were isolated from about 100 mg of pancreas or liver by Dounce homogenization followed by centrifugation essentially as described (Mulvihill and Palmiter 1977), except that 1 mM EGTA was added to buffers NA, NB, and NC. Nuclei were stored in buffer NC at −70°C and thawed on ice for transcription and DNase I hypersensitivity assays.

Nuclear transcription assays. Transcription assays were performed as previously described (McKnight and Palmiter 1979), except that nitrocellulose filter discs for hybridization contained about 4 μg of plasmid DNA and hybridizations were incubated at 45°C for 36 hours. Individual filters were washed as previously described (McKnight and Palmiter 1979) and counted for

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10 minutes in a Packard Liquid Scintillation spectrom-
eter. Transcription rates (expressed in ppm) were cal-
culated by dividing hybridized cpm minus background
by the length of the cloned probe in kilobases and by
the input counts of ^{32}P-labeled RNA.

DNase I hypersensitivity assays. DNase I hyper-
sensitivity studies were carried out by adding 0.1 μg/ml
to 5 μg/ml DNase I in 5 mM MgCl$_2$ and 1 mM CaCl$_2$ to
approximately 20 μg of nuclear DNA in buffer NC.
Digestion was carried out at 37°C for 10 minutes with
pancreas nuclei, and at 25°C for 5 minutes with liver
nuclei. Nuclei were then incubated in 1% SDS and 100
μg/ml proteinase K for 1 hour at 37°C followed by ex-
traction with phenol/chloroform and then chloro-
form. Total nucleic acids were precipitated in 70%
ethanol and 0.1 M NaCl. Nucleic acids were dissolved
in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5), treated with
RNase A, and digested with the indicated restriction
enzymes and analyzed by Southern blotting.

Histology. Tissue samples were fixed overnight in
Bouin's fixative. Tissue samples were imbedded in par-
affin by standard procedures, and 6-μm sections were
stained with hematoxylin and eosin.

Immunoperoxidase staining. Tissues were fixed
overnight in Bouin's fixative, washed in 70% ethanol,
and embedded in paraffin. Six-micrometer sections
were mounted on glass slides and stained for SV40 large
T antigen by an indirect immunoperoxidase procedure,
using the monoclonal antibody 412 (Gurney et al. 1980)
and the ABC avidin-biotin system (Vector Laborato-

ries). Sections were exposed to a 1:100 dilution of 412
culture supernatant (gift from A.J. Levine, Princeton
University) overnight at 4°C, treated with the ABC re-
agents, and then incubated for 5 minutes in 0.3 mg/ml
diaminobenzidine, 0.01% H$_2$O$_2$, and 2% nickel am-
monium sulfate.

RESULTS AND DISCUSSION

Pancreas-specific Expression of Elastase-hGH Fusion Genes

Transgenic mice containing elastase-hGH fusion
genes with either 4.5 kb, 0.5 kb, or 0.2 kb of elastase 5'-
flanking sequence (Fig. 1b) expressed hGH mRNA only
in the pancreas. In most cases we examined eight tis-
sues: intestine, kidney, liver, pancreas, parotid gland,
spleen, submandibular gland, and testes or ovary. We
have never observed significant expression in a non-
pancreatic tissue. Analysis of 21 transgenic mice con-
taining these three fusion genes (Table 1) reveals that
hGH mRNA levels are up to three to four orders of
magnitude higher in the pancreas than in other tissues
in 15 out of 21 mice. Five mice failed to express the
foreign genes in any tissue and one mouse had very
low-level expression in the pancreas. Some mice in each
group expressed more hGH mRNA than endogenous
elastase mRNA (about 10,000 molecules/cell; Swift et
al. 1984a), suggesting that elastase sequences can func-
tion in both a quantitatively and qualitatively normal
fashion when inserted into random chromosomal po-
sitions.

Table 1. Expression of Elastase-hGH Genes in Tissues of Transgenic Mice

| Plasmid | Mouse[a] | Genes/cell[b] | hGH mRNA[c] | |
| | | | pancreas | other tissues |
			(molecules/cell)	
E4.5hGH	37-9	6.5	<10	<10
	31-7	2.2	35	<10
	34-4	1.5	1,170	<10
	34-9	3.2	2,560	<10
	34-2	1.4	4,000	<10
	35-1	1.2	8,490	<10
	33-6	6.2	15,100	<10
	34-10	6.2	28,100	<10
E0.5hGH	43-4*	1.0	<10	<10
	43-10*	6.0	<10	<10
	42-6*	1.2	1,460	<10
	44-6*	199.0	9,760	<10
	44-4*	136.0	11,900	<10
	43-5*	2.3	18,000	<10
	40-2*	4.2	39,400	<10
E0.2hGH	47-4	3.3	<10	<10
	48-4	39.4	<10	<10
	49-3	2.1	3,340	<10
	136-2*	3.4	16,100	<10
	132-5*	3.4	33,100	<10
	129-3*	3.4	51,000	<10

[a]*, These mice received elastase-hGH genes lacking vector sequences.
[b]Determined by quantitative DNA dot hybridization (Ornitz et al. 1985).
[c]Determined by solution hybridization with a 21-base oligonucleotide probe. (See Exper-
imental Procedures.)

Our previous data indicated that elastase-hGH fusion genes yield an hGH mRNA of normal size which is translated into an immunoreactive hGH protein that is secreted into the pancreatic ducts along with the digestive enzymes (Ornitz et al. 1985). Immunohistology was used to demonstrate that hGH was located exclusively in all acinar cells of the pancreas (Ornitz et al. 1985). Furthermore, all transgenic mice containing elastase-hGH fusion genes grow normally. This observation indicates that these fusion genes are not expressed in any cells that secrete into the bloodstream. If hGH were expressed in such cells, then we would expect to observe an increased growth rate in these mice as is observed when metallothionein (MT)-hGH fusion genes are expressed in transgenic mice (Palmiter et al. 1983). Thus, the lack of enhanced growth, the lack of hGH mRNA in nonpancreatic cells, and the exclusive immunofluorescence of hGH in the acinar cells of the pancreas argue that the 213 bp of elastase sequences present in E0.2hGH are sufficient to direct expression of hGH to pancreatic acinar cells.

Analysis of additional deletion mutants and constructs in which the elastase region is inverted or moved further away will be necessary to establish the precise cis-acting sequences involved in pancreas-specific expression and to determine if this region has enhan-

cer-like properties. The 213-bp elastase I sequence contains a 37-bp region that is conserved between the elastase I and II genes; it is also homologous to similarly positioned sequences in the chymotrypsin gene and two trypsin genes (Swift et al. 1984b). Walker et al. (1983) have shown that this region is essential for expression of chymotrypsin-CAT fusion genes in cultured acinar cells.

Elastase hGH Pedigrees

We have established two elastase-hGH lines to determine whether tissue-specific expression is transmissible and whether the level of expression is relatively stable from one offspring to another. Pedigree 43-5 shows three generations of mice containing the E0.5hGH genes (Fig. 2a). The founder mouse transmitted the E0.5hGH genes to only 2 out of 10 of its progeny, whereas in the F₁ generation these genes were transmitted normally (6/12). The average amount of hGH mRNA in the offspring was 63,000 molecules/cell compared with 18,000 in the founder. These observations suggested that the founder was probably mosaic. Consistent with this interpretation, quantitative DNA blot hybridization revealed that the founder mouse contained an average of 2.3 copies of E0.5hGH per cell,
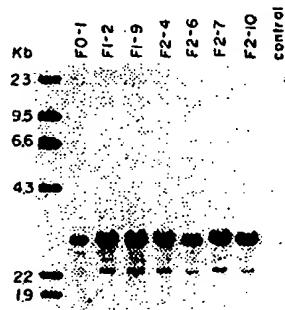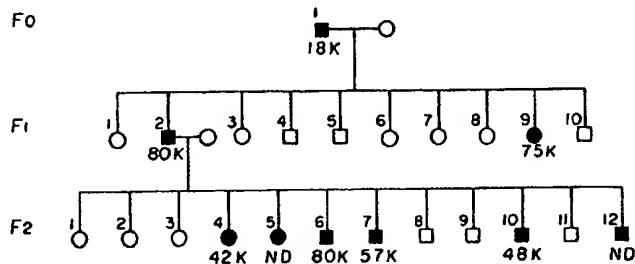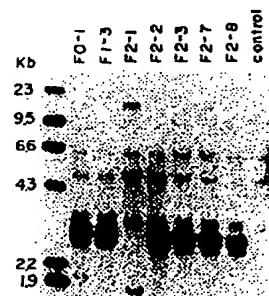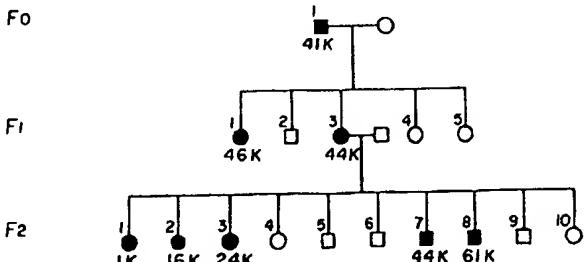
Figure 2. Elastase-hGH pedigrees (left). Squares represent males, circles represent females; solid symbols represent animals containing E0.5hGH fusion genes. The numbers below the symbols indicate hGH mRNA levels in thousands of molecules per cell. The Southern blots (right) are SstI digests of kidney DNA. In a the blot was probed with a nick-translated hGH structural gene fragment. In b the blot was probed with a MT-hGH fusion gene fragment (the bands at 6.0, 4.5, and 2.9 kb represent endogenous MT gene fragments).

whereas its progeny contained about seven genes per cell (data not shown). Furthermore, a Southern blot of DNA digested with Ss*t*I, an enzyme that cuts once within the 2.8-kb piece of DNA that was introduced into this mouse, yielded a prominent band of 2.8 kb, indicative of a tandem head-to-tail array, plus fainter junction fragments (Fig. 2a). The intensity of the bands in the offspring is significantly greater than the founder, in agreement with the quantitative dot analysis, but the pattern is identical in all of the mice.

A second pedigree, 40-2, is shown in Figure 2b. These mice carry about four copies of E0.5hGH genes and the genes appear to be transmitted in a normal Mendelian manner as though all the genes were closely linked on one chromosome. Southern blot analysis of Ss*t*I-digested DNA (Fig. 2b) indicates a major 2.8-kb fragment plus several junction fragments; the pattern and intensity of the bands are similar in all of the mice except one mouse in the F_1 generation (F2-1) that is missing the tandem repeat and has acquired several new bands. This mouse also has an unusually low level of hGH mRNA in the pancreas. Apparently, a DNA rearrangement occurred during meiosis of the egg that gave rise to this mouse. (The bands at 6.0, 4.5, and 2.9 kb represent endogenous MT DNA fragments that were probed as an internal control.)

Both pedigrees reveal that elastase-hGH fusion genes are in the germ line and can be stably (with one notable exception) transmitted through several generations.

Expression of the fusion genes is also reasonably constant. Mice from these lines were used to study transcription and chromatin structure of the elastase fusion genes (see below). These lines will also be useful for studying developmental regulation of elastase-hGH gene expression.

Transcriptional Activity of the Elastase Fusion Genes

To ascertain whether the elastase regulatory region is capable of conferring quantitatively normal transcription as well as tissue specificity, we measured the rate of elastase-hGH transcription relative to the endogenous elastase genes. This approach avoids problems associated with mRNA and/or protein accumulation which might be subject to differential stability.

Nuclei were isolated from pancreas and liver of offspring from transgenic line 43-5 containing seven tandem copies of the E0.5hGH gene. These nuclei were allowed to continue transcriptional elongation in the presence of [α-32P]UTP in a so called nuclear "run-on" assay. Specific 32P-labeled RNA transcripts were quantitated by hybridizing them to nitrocellulose-bound plasmid DNA containing homologous cloned sequences of the hGH gene, the mouse elastase I gene, the liver albumin gene, or pBR322 plasmid DNA. The slope from a plot of input 32P-labeled RNA versus hybridized specific RNAs (Fig. 3) gives a value that represents relative transcription rates. These values are ex-
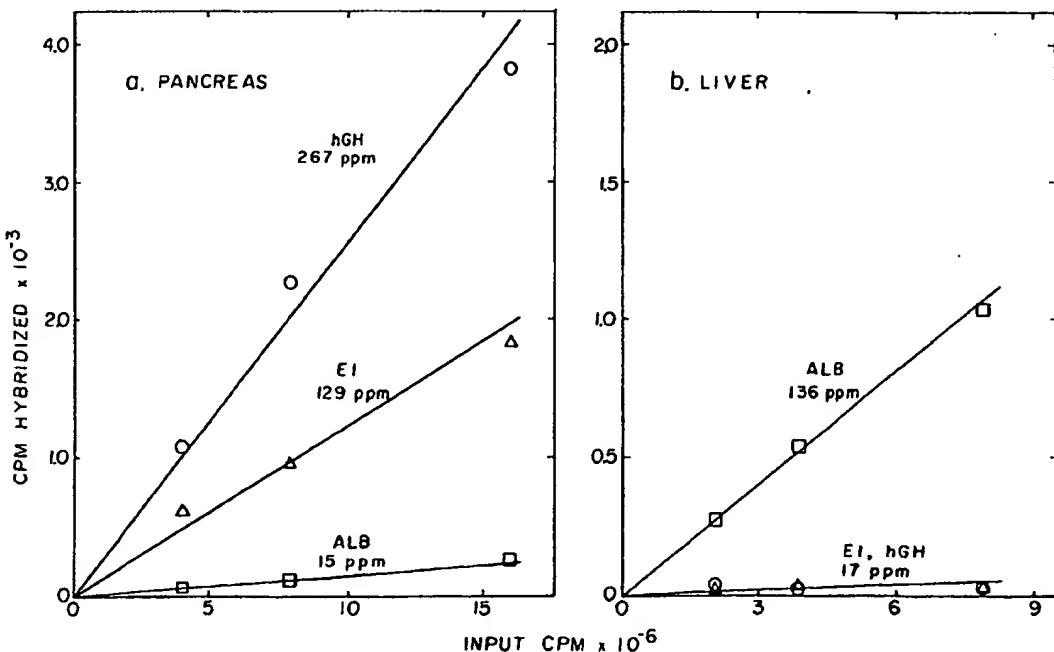
Figure 3. Relative rate of transcription of elastase, E0.5-hGH, and albumin genes in pancreas and liver nuclei from mice of the 43-5 line. Nuclei were isolated and endogenous RNA polymerases were allowed to elongate products started in vivo as described under Experimental Procedures. Labeled RNA transcripts were hybridized to immobilized plasmid DNA containing sequences corresponding to hGH (O), elastase (△), and albumin genes (□). (*a*) Hybridization of RNA synthesized in pancreas nuclei; (*b*) a similar experiment using liver nuclear RNA. The relative rate of transcription in ppm was calculated as described in Experimental Procedures.

pressed in ppm after correcting for probe lengths and background, as described in Experimental Procedures.

We calculate that in the pancreas of mice from line 43-5 there are about two times more RNA polymerase molecules engaged in E0.5hGH gene transcription than in endogenous elastase I gene transcription (Fig. 3a). This apparent increased transcription rate may be attributable to the seven tandem copies of the E0.5hGH gene in this transgenic line compared with the two endogenous elastase I genes. Because there is little relationship between gene copy number and mRNA accumulation in transgenic mice (see Table 1), we do not know whether all seven copies of the elastase-hGH fusion genes are transcribed equally well. Nevertheless, we can set some limits: If only one gene is functional, then it is transcribed at about four times the rate of an endogenous elastase gene, and if all seven are transcribed, then they are transcribed at about half the rate of the endogenous elastase genes. Either way, the results suggest that the foreign elastase promoter is recognized about as efficiently as the endogenous promoters, despite being in a completely foreign chromosomal environment.

Transcription of the E0.5hGH gene and the mouse elastase I gene was very low in liver nuclei (Fig. 3b), indicating that the tissue-specific expression is due predominantly, if not exclusively, to transcriptional specificity. Conversely, the albumin gene is transcribed at a high level in the liver and not in the pancreas. This gene serves as a control for functional liver nuclei and also demonstrates a background transcription rate in the pancreas, similar to that of elastase and hGH in the liver. We believe that the background rate of 15–17 ppm seen for all three genes is due to nonspecific binding to our plasmid probes.

The Elastase Sequence Is Associated with a DNase I Hypersensitive Site

DNase I hypersensitive sites are frequently associated with actively transcribed structural genes (Stalder et al. 1980; Sweet et al. 1982; Senear and Palmiter 1983; Becker et al. 1984; Kunnath and Locker 1985). These sites are often at the 5′ end of the gene and may reflect perturbations of chromatin structure due to sequence-specific DNA binding proteins. These hypersensitive sites have been shown to correspond with tissue-specific and developmental regulatory elements. For example, the immunoglobulin genes have hypersensitive sites in the intron between the VDJ and C regions in B-cell lines (Parslow and Granner 1982), the insulin gene has a hypersensitive site in its 5′ region specifically in β cells (Wu and Gilbert 1981), and the β-globin gene develops a hypersensitive site in its 5′ region when mouse erythroleukemia cells are induced to differentiate (Miller et al. 1978). The hypersensitive sites in the murine mammary tumor virus (MMTV) long terminal repeat (LTR) correlate with transcriptional activation of the MMTV promoter by the activated glucocorticoid receptor (Zaret and Yamamoto 1984).

Here we show that a hypersensitive site is established near the elastase regulatory element in the chromatin from pancreas of mice from the 43-5 line. This mouse line carries seven tandem copies of E0.5hGH (NdeI restriction fragment from Fig. 1b). The map of this integrant is shown in Figure 4a. Digestion with BglII yields a 2.84-kb internal BglII fragment and a 12-kb band including the junction between mouse DNA and the insert. These bands are shown in Figure 4b (lane 1, no DNase I treatment or incubation). To detect hypersensitive sites near the 5′ end of these genes, we purified DNA from DNase I-digested nuclei, restricted with BglII, and hybridized a Southern blot of this DNA with a probe derived from the middle region of the hGH gene (as shown in Fig. 4a). Digestion of pancreas nuclei with DNase I revealed a band of about 1.4 kb (lanes 2-9) that was not present when liver nuclei were digested (lanes 2-5). The band of approximately 1.4 kb maps a hypersensitive site to the vicinity of the elastase promoter (see Fig. 4a). Additional experiments with more precise size markers (SalI-BglII- and BamHI-BglII-digested DNA) indicate that the hypersensitive site maps to the region between −150 and +50 of the E0.5hGH fusion gene. Liver nuclei show no hypersensitive sites in the region of the elastase promoter element, but they do show a uniform 1-kb band present in all lanes that is probably due to digestion by endogenous nucleases during nuclei isolation.

This experiment demonstrates that the elastase sequence generates a DNase I-sensitive region in mouse pancreatic chromatin in close proximity to the tissue-specific regulatory region and promoter. Because the integration site of the E0.5hGH genes is probably random in transgenic mice, this experiment also demonstrates that the ability to form a tissue-specific hypersensitive site is independent of precise chromosomal location. Furthermore it is independent of the elastase I structural gene. It remains to be demonstrated whether the elastase regulatory element can yield a hypersensitive site in the absence of a functional transcription unit.

Pancreatic Carcinomas Induced by Elastase-SV40 T Antigen

The early region of the SV40 virus codes for two proteins: large and small T antigens. These proteins affect the expression of viral and cellular genes and can lead to cell transformation (Tooze 1980). Introduction of SV40 early-region genes into mice resulted in a high frequency of choroid plexus tumors (Brinster et al. 1984). Further experiments demonstrated that large T antigen was sufficient for tumorigenesis and that the SV40 enhancer (72-bp repeats) was required for development of choroid plexus tumors (Palmiter et al. 1985). When the SV40 early region was fused to the 5′-flanking region of the rat insulin gene and introduced into mice, the transgenic mice expressed T antigen only in the β cells and ultimately developed β-cell tumors (Hanahan 1985). Thus, it appears that the site of tu-

a. TRANSGENIC MOUSE 43-5 F2-5

b.

PANCREAS

LIVER
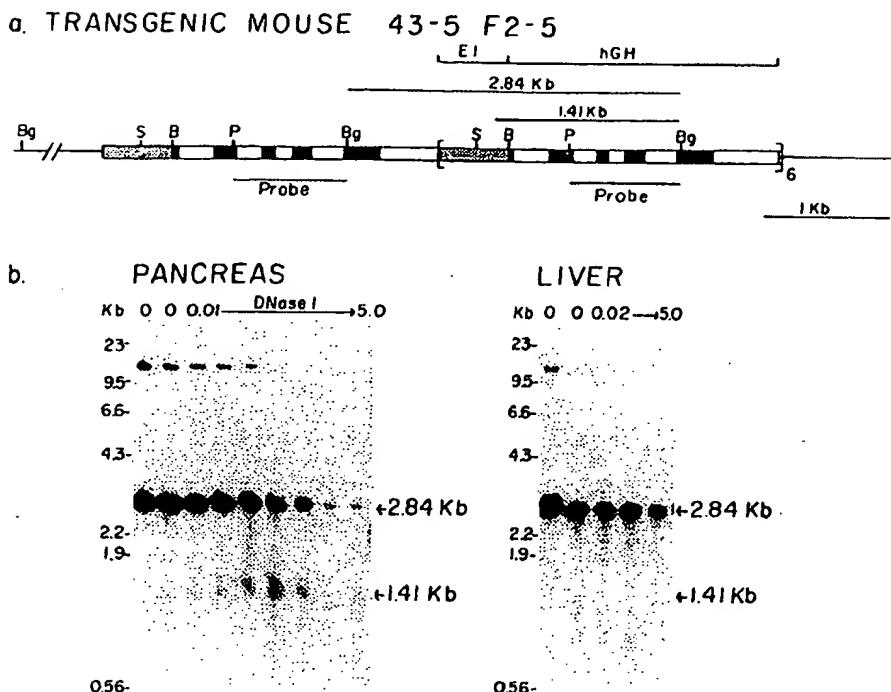
Figure 4. Analysis of DNase I hypersensitivity sites in E0.5hGH genes from line 43-5. (a) Map of the insertion containing seven tandem copies of the E0.5hGH gene. The elastase sequences are stippled; the hGH exons are solid. The inserted DNA was 2.84 kb and gives a prominent band of this size when cut with BglII, an enzyme that cut once within the insert. The 1.41-kb line marks the location of the band that appears after nuclease digestion. (B) BamHI; (Bg) BglI; (P) PvuII; (S) SalI. (b) Southern blot analysis of DNase I-treated nuclei. Pancreas nuclei were digested at 37°C for 10 min and liver nuclei were digested at 25°C for 5 min; DNase I concentrations ranged from 0 to 5 μg/ml; one sample (next to markers) was not incubated. After digestion, the DNA was purified, digested with BglII, electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with a nick-translated PvuII-BglII DNA fragment shown in a.

morigenesis can be directed to specific cell types by using different transcriptional regulatory elements.

The foreign gene that we injected contained 7.2 kb of elastase 5'-flanking sequence fused to the SV40 T antigen structural gene (Fig. 1c). Five mice containing this fusion gene were produced. These mice were bred to start transgenic lines and then observed for the development of any pathological symptoms. Transgenic line 177-5 was studied in detail (Fig. 5, Table 2). All of the mice in the F₁ generation developed large abdominal masses at 3–7 months of age and died within a few weeks of obvious abdominal swelling. Mice in the F₂ generation are approaching the age when tumors developed in their parent.

The abdominal masses that originated in the pancreas were firm and lobular and generally grew rapidly to enormous size. The mouse in Figure 6A had a 9.1-g tumor when sacrificed. These tumors occasionally spread by transcoelomic seeding. Rare metastases to liver and lung were also observed. Histological examination revealed nearly complete replacement of normal pancreatic tissue (Fig. 6B) by sheets of pleomorphic epithelial cells (Fig. 6C), many of which formed pseudoacinar structures, with numerous mitoses. SV40 large

T antigen was detected immunocytochemically in the nucleus of most tumor cells (Fig. 6D).

To assess the relationship between T antigen gene expression and tumorigenesis, we analyzed pancreatic sections from mice of different ages that inherited the elastase T antigen genes. Sections taken between 4 and 28 days after birth revealed a few isolated cells in the exocrine pancreas that were positive for large T antigen (Fig. 6E); tissue morphology at this stage was essentially normal. Between 4 and 8 weeks the isolated foci increased in size, possibly by clonal expansion (Fig. 6F). The earliest pathological changes evident by hematoxylin and eosin staining were an increase in size and irregularity of the acinar cell nuclei. The first histologic evidence of actual tumor formation was an expanding growth of disorganized acinar epithelial cells with numerous mitoses (Fig. 6G). These cells showed more intense staining for T antigen (Fig. 6H), but there was marked cell-to-cell variation even within such a focus.

We also measured the SV40 mRNA in pancreas from transgenic mice of different ages (Fig. 5, Table 2). In young mice, SV40 mRNA was either undetectable or very low. By 11 weeks, SV40 mRNA was generally detectable but the amount increased sharply in overt tu-

E-SV40   177-5 PEDIGREE
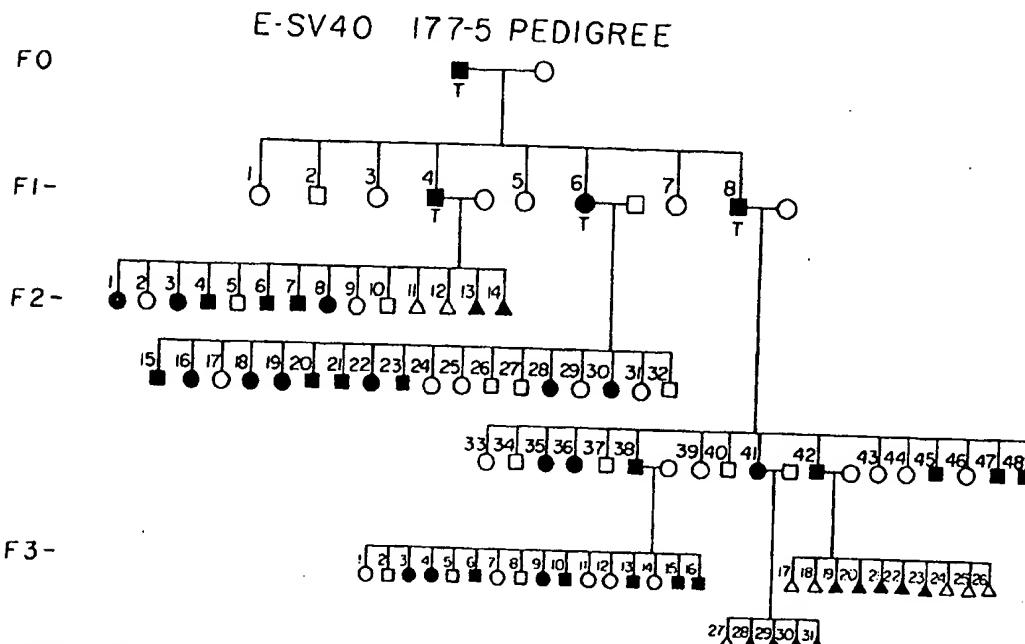
Figure 5. Pedigree of the 177-5 line of mice carrying E7.2SV40 T antigen fusion genes. Symbols are the same as in Fig. 2 legend. T signifies that a pancreatic tumor developed; triangles indicate mice where sex was not determined.

Table 2. Pathology and Gene Expression in Pancreatic Tumors of Transgenic Mice

Mouse[a]	Age[b] (days)	Histology[c]	Pancreatic mRNA content		
			elastase I[d] (% normal pancreas)	amylase[d]	T antigen[e] (molecules/cell)
F1-4	210	+ + +	3.4	3.8	269
F2-4	140	+ + +	59.0	70.3	25
F2-38	108	+ + +	27.8	44.0	13
F2-41	108	+ + +	39.6	59.0	16
F2-47	87	+ +	75.7	87.1	12
F2-48	87	+ +	93.4	100.0	14
F3-19	26	+	ND	ND	<5
F3-20	26	+	ND	ND	<5
F3-28	19	+	ND	ND	<5
F3-29	19	+	ND	ND	<5
F3-30	19	+	ND	ND	<5
F3-31	19	+	ND	ND	<5
F3-21	4	−	ND	ND	<5
F3-22	4	+	ND	ND	<5
F3-23	4	−	ND	ND	<5
261-4	80	+ + +	9.1	23.2	11
264-4	140	+ + +	34.1	20.1	22
266-5	200	+ + +	3.6	3.1	12
266-6	112	+ + +	10.5	15.7	40

[a]Individual mice in the F_1, F_2, and F_3 generations from pedigree 177-5 are identified in Fig. 5.
[b]The age when mice either died of pancreatic tumors or were sacrificed for tissue analysis.
[c]+ + +, Large tumors; + +, focal tumors; +, isolated cells or very small T antigen-positive foci; −, no T antigen-positive cells.
[d]mRNA levels were determined by solution hybridization of total nucleic acid (TNA) with ^{32}P-labeled oligonucleotides specific for amylase and elastase mRNA. The values are presented relative to normal pancreas.
[e]T antigen mRNA was quantitated by solution hybridization with an oligonucleotide specific to the 3' end of the T antigen gene. Molecules per cell were calculated by using an M13 standard, estimating the size of T antigen mRNA, and determining the RNA/DNA ratio of the TNA samples (Durnam and Palmiter 1983).

406
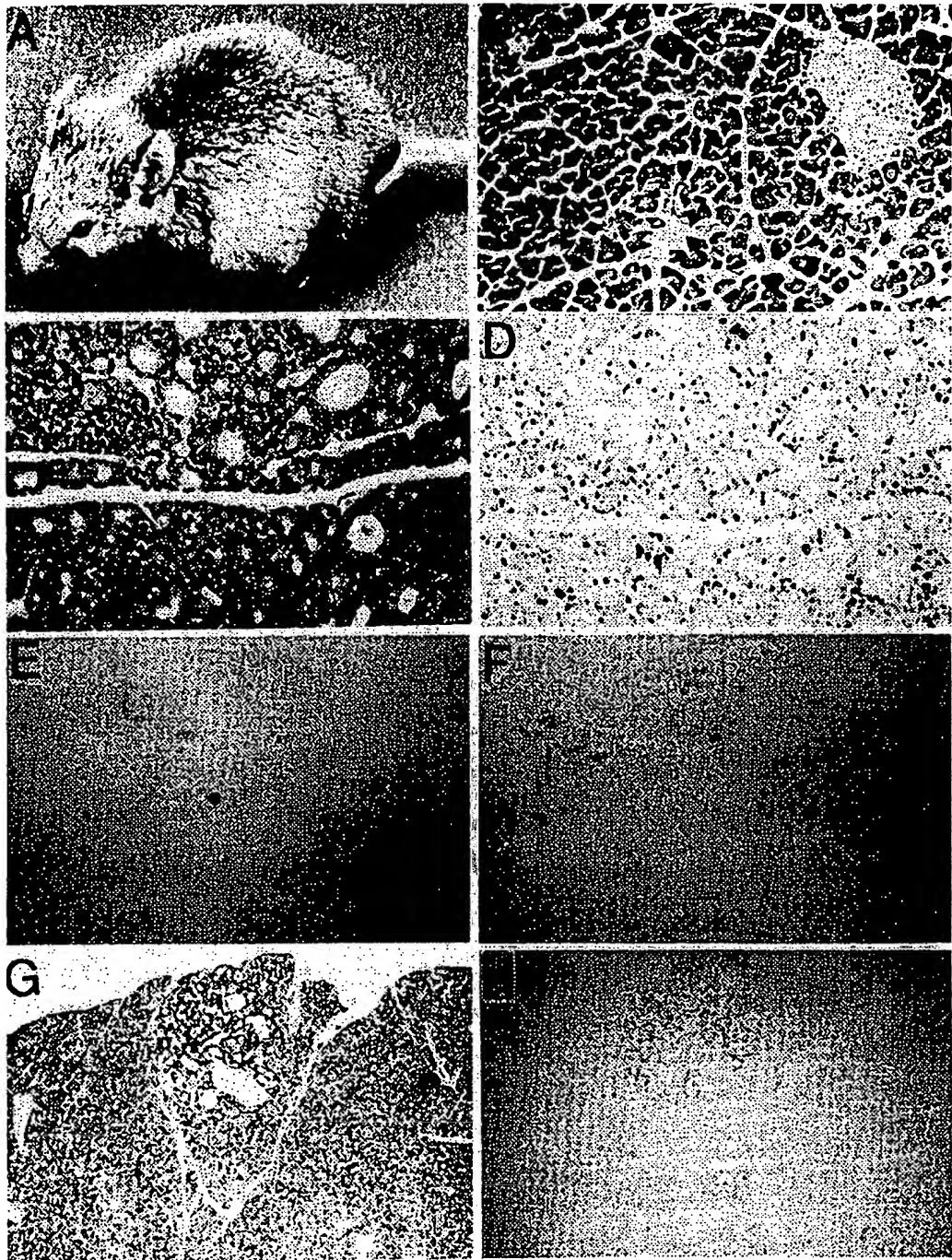
Figure 6. Histology of mice containing elastase-SV40 T antigen fusion genes. (*a*) Transgenic mouse with abdominal swelling (this mouse had a 9.1-g pancreatic tumor). (*b*) Hematoxylin and eosin-stained section of normal pancreas. (*c*) Hematoxylin and eosin-stained section of a pancreatic tumor (mouse F1-4; Fig. 5). (*d*) Indirect immunoperoxidase staining for T antigen of the pancreatic tumor shown in *c* (280-day-old mouse). (*e*) Immunoperoxidase staining for T antigen in pancreas of a 26-day-old mouse (F3-22). (*f*) Immunoperoxidase staining for T antigen in pancreas of a 87-day-old mouse (F3-16). (*g*) A focal tumor in the pancreas of mouse F3-16 visualized with hematoxylin and eosin. (*h*) Immunoperoxidase staining for T antigen in the focal tumor shown in *g*.

mors. These results corroborate the immunological staining for SV40 T antigen and suggest that the increased expression is due to proliferation of a subpopulation of cells expressing T antigen. We also noted that as the expression of T antigen increased there was a progressive decrease in the amount of elastase and amylase mRNA (Table 2).

The results obtained with elastase SV40 T antigen genes are quite different from those observed with elastase-hGH. In the latter case, all the acinar cells expressed hGH and at very high levels, whereas T antigen expression appears to commence in a small number of acinar cells and then increase presumably by clonal expansion. These results also contrast with those obtained by Hanahan (1985) with a comparable insulin T antigen construct. He observed relatively uniform expression of T antigen in all β-cells that was associated with hypertrophy of those cells; eventually some of those cells proliferated and formed overt tumors.

We do not know what causes the activation of SV40 T antigen gene expression in selected pancreatic acinar cells. The availability of lines of mice that routinely develop pancreatic tumors (Fig. 5) will allow a more detailed analysis of the molecular events associated with tumor development.

Pancreatic tumor tissue from mouse 266-6 was used to start a cell line. This cell line has been maintained in culture for several months. The cells grow in clumps (resembling acini) and they express high levels of SV40 mRNA (450 molecules/cell); this value is considerably higher than in the primary tumor (Table 2). This cell line retained about 2% of normal elastase mRNA, but amylase mRNA was barely detectable. Because these cells contain an elastase-SV40 fusion gene, we suspect that there is selective pressure to maintain some degree of differentiation. Failure to maintain the proteins involved in elastase promoter function would presumably lead to cessation of the transformed phenotype.

CONCLUSIONS

To identify the cis-acting DNA sequence elements required for pancreas-specific expression of the rat elastase I gene, the 5'-flanking region of the elastase I gene was joined to the hGH structural gene. Elastase-hGH fusion genes with 4.5, 0.5, and 0.2 kb of elastase 5'-flanking sequence were introduced into mice. Most of the mice carrying these three constructs expressed high levels of hGH mRNA in the pancreas but undetectable levels in other tissues. The data indicate that the sequence between −205 and +8 bp of the elastase promoter is sufficient to direct expression of hGH exclusively to the pancreatic acinar cells. Two lines of mice were established that transmit tissue-specific expression of hGH in a stable manner. Nuclei isolated from pancreas or liver of these mice were used to demonstrate that transcription of the elastase-hGH gene is tissue specific and quantitatively similar to the endogenous elastase genes. These nuclei were also used to demonstrate that there is a DNase I hypersensitive site

located in the elastase regulatory element in pancreatic nuclei but not in liver nuclei. The results suggest that sequences in the close proximity to the elastase promoter bind a specific protein(s) and this leads to transcriptional activation exclusively in the acinar cells of the pancreas. The function of this sequence is apparently independent of chromosomal location.

When the elastase 5' sequences were fused to SV40 T antigen genes, large pancreatic tumors routinely developed at 3–7 months of age in transgenic founder mice and their offspring. Development of these tumors appears to reflect activation of SV40 T antigen expression in a small number of acinar cells, followed by their proliferation into overt tumors rather than high-level expression in all acinar cells, as was observed when this regulatory element was fused to the hGH gene.

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An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice

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Transgenic mice were used to locate the cis-acting DNA elements that are important for efficient, tissue-specific expression of the mouse albumin gene in the adult. Chimeric genes with up to 12 kb of mouse albumin 5'-flanking region fused to a human growth hormone (hGH) reporter gene were tested. Remarkably, a region located 8.5–10.4 kb upstream of the albumin promoter was essential for high-level expression in adult liver and the region in between −8.5 and −0.3 kb was dispensable. The far-upstream region behaved like an enhancer in that its position and orientation relative to the albumin promoter were not critical; however, it did not function well with a heterologous promoter. Two of four DNase hypersensitive sites found in the 5'-flanking region of the albumin gene map to the far-upstream and promoter regions; the others may reflect regions involved in developmental or environmental control of this gene.

The albumin and α-fetoprotein genes are evolutionarily related and separated by 14 kb on mouse chromosome 5 (Ingram et al. 1981). Furthermore, they are developmentally regulated in a similar fashion in that they are induced coordinately in fetal liver, yolk sac, and gastrointestinal tract (Krumlauf et al. 1985a). However, albumin expression is maintained in the adult liver whereas α-fetoprotein expression declines to undetectable levels shortly after birth; both genes are repressed in nonhepatic tissues of the adult. They are regulated primarily at the transcriptional level and their mRNAs are among the most abundant polymerase II transcripts in the liver and code for the most abundant serum proteins of fetus and of adult (Tilghman and Belayew 1982).

Progress has been made in dissecting the cis-acting DNA elements involved in the developmental and tissue-specific control of α-fetoprotein gene expression by assaying various constructs in transgenic mice (Krumlauf et al. 1985a,b; Hammer et al. 1987) or after transfection into tissue culture cells (Godbout et al. 1986; Muglia and Rothman-Denes 1986; Widen and Papaconstantinou 1986). When tested in transgenic mice, a construct with 7 kb of 5'-flanking region associated with

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an α-fetoprotein minigene allowed proper tissue-specific expression at levels exceeding those of the endogenous genes in some cases (Hammer et al. 1987), suggesting that all of the elements necessary for proper expression lie within that construct. Further dissection revealed that the α-fetoprotein promoter and 5' sequences extending to −1 kb did not promote expression in any tissue; however, when it was combined with any of three large regions that cover the region from −1 to −7 kb, there was substantial, but not equivalent, expression in all three target tissues. In addition to appropriate tissue-specific expression, the α-fetoprotein gene was repressed after birth and activated by liver damage in the adult, in concert with the endogenous genes (Hammer et al. 1987). In contrast to the results in transgenic mice, the α-fetoprotein promoter does stimulate expression in a cell-specific manner when transfected into cultured cells, and a region located between −52 and −85 appears to be essential for this property (Godbout et al. 1986; Muglia and Rothman-Denes 1986; Widen and Papaconstantinou 1986). Each of the upstream regions behaved like a typical enhancer (Serfling et al. 1985) in that its orientation and position relative to the promoter could be changed. Furthermore, they could activate a heterologous promoter, when tested by transfection into cultured liver cells (Godbout et al. 1986).

Previous studies, in which the rat albumin promoter

and 5'-flanking region were tested by transfection or infection into hepatoma cells, revealed that there is an important, tissue-specific element located within 400 bp of the cap site (Ott et al. 1984; Friedman et al. 1986). Furthermore, the mouse albumin promoter is transcribed more efficiently in cell-free extracts prepared from liver than from other tissues (Gorski et al. 1986). Using this in vitro transcription assay, the DNA elements required for preferential expression in liver extracts were localized to a region between 55 and 170 bp upstream of the cap site (Gorski et al. 1986). However, previous studies have not addressed the question of whether there is an albumin enhancer as well.

Considering that the albumin and α-fetoprotein genes are related, linked, and regulated similarly, one might expect the organization of the regulatory domains to be conserved. Therefore, we set out to ascertain whether the albumin gene has enhancers and, if so, where they lie in relation to the structural gene. We show here that the albumin promoter is tissue-specific in transgenic mice and there appears to be only one region with enhancer-like activity that is located much further upstream than any of the α-fetoprotein gene elements.

Results

Albumin-growth hormone fusion genes are expressed in liver

Because we wished to examine the control of the mouse albumin gene in transgenic mice, we selected human growth hormone (hGH) as a heterologous reporter gene. This choice was based on the facts that the hHG gene has been successfully used in combination with a number of other regulatory regions, its mRNA appears to be relatively stable in a variety of cells, and it stimulates growth if sufficient amounts of GH are secreted into the bloodstream (Palmiter et al. 1983; for review, see Palmiter and Brinster 1986). To construct albumin–human growth hormone (alb–hGH) fusion genes, a synthetic BamHI linker was inserted 22 bp downstream from the albumin cap site in an albumin subclone that spans the promoter region (see Materials and methods). This subclone was then fused to the hGH gene at its natural BamHI site at +3. Thus, the albumin promoter and 5'-flanking region were joined to the hGH structural gene near the beginning of the first exon of each gene such that the transcription start site would be determined by albumin sequences and the resulting hGH mRNA would have 22 extra nucleotides contributed by albumin sequences. Additional albumin 5'-flanking region was inserted into this clone to create a fusion gene with about 12 kb of albumin 5' sequence fused to the hGH gene (Fig. 1). The proper reconstruction of the albumin sequences was verified by Southern blot comparison of the plasmid clone and genomic DNA after digestion with several enzymes.

Restriction fragments containing about 0.3, 0.9, 3.8, 8.5, or 12 kb of albumin 5'-flanking region and the hGH structural gene (top line, Fig. 1) were separated from the remaining plasmid DNA by agarose gel electrophoresis.

A few hundred copies of each of these DNA fragments were microinjected into pronuclei of fertilized eggs to produce transgenic mice (Brinster et al. 1985). Groups of 3–21 transgenic mice were produced with each of these constructs. With the exception of those mice containing the 0.3 alb–hGH construct, some mice grew significantly larger than normal, indicating that the alb–hGH genes were being expressed in some tissues of those mice.

To determine in which organs the genes were being expressed, the mice were sacrificed and hGH mRNA levels were measured in a variety of organs by a solution hybridization assay using an oligonucleotide complementary to a sequence in exon 4 of hGH mRNA. hGH mRNA was detected in livers of all mice that grew larger than normal as well as in a few other mice in each group; it was also detected in the kidneys of a few mice, but not in any other tissue that was assayed, including spleen, intestine, pancreas, brain, heart, muscle, lung, and testis. Figure 2 shows the levels of hGH mRNA detected in liver and kidney of each of the founder mice and indicates which mice grew larger than normal. The mean level of hGH mRNA in the liver of those mice that expressed the transgene is indicated by the histograms. The frequency of detecting expression was low (only 2 of 10 transgenic mice), with the construct containing only 0.3 kb of albumin sequence, but was high for the constructs with 0.9, 3.8, 8.5, and 12 kb of albumin sequence (Fig. 2). The extreme variability in the level of expression from one mouse to another is often observed in transgenic mouse experiments and probably reflects influences from the chromosomal site of integration, as well as variable transgene copy number and mosaicism of some of the founders (Palmiter and Brinster 1986).

Although there was a progressive increase in the average level of hGH mRNA detected in the liver as more albumin-flanking DNA was included in the constructs (Fig. 2), the level of hepatic expression was dramatically higher in most of the mice with 12 kb of albumin sequence. The average level of hGH mRNA in these mice was about 5000 molecules/cell and in some mice the level was comparable to that of endogenous albumin mRNA, estimated to be about 10,000 molecules/cell (Tilghman and Belayew 1982). These data suggest that there is an enhancer-like element located 8.5–12 kb upstream of the promoter. Moreover, the albumin promoter, which is contained within the 0.3-kb construct, is tissue-specific but it has a propensity not to be expressed unless additional 5' sequence is present, as in the 0.9-, 3.8-, and 8.5-kb alb–hGH constructs. Curiously, the constructs with 0.3–8.5 kb of albumin sequence also promoted a low level of expression in the kidney of some mice. However, this activity appears to be extinguished when the enhancer-like element between −8.5 and −12 kb is present, since no kidney expression was detected in any of the mice with the 12-kb construct (Fig. 2).

Because many of the mice grew larger than normal, we knew that functional hGH mRNA was produced, indi-

mouse albumin- human growth hormone
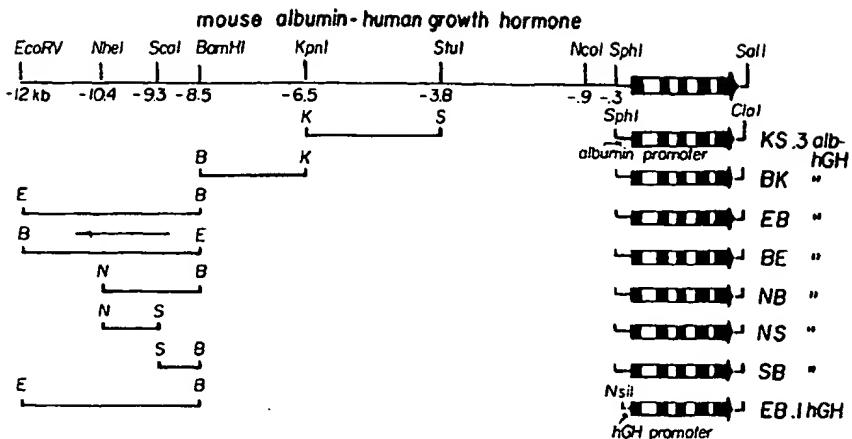
Figure 1. Maps of the alb–hGH gene constructs tested in transgenic mice. The 12-kb alb–hGH construct (top line) was prepared as described under Materials and methods. The 12-, 8.5-, 3.8-, 0.9-, and 0.3-kb alb–hGH DNA fragments were isolated using unique sites in the albumin sequence and the SaII site at the 3' side of the hGH gene. The internal deletions were created as described under Materials and methods; in each case the upstream albumin fragments were joined to the albumin promoter at the SphI site that lies at −0.3 kb; the letter designations refer to the restriction enzymes used to isolate the albumin fragments. The EB 0.1–hGH construct has the albumin EB fragment joined to an NsiI site located at −90 in the hGH gene. Each of these latter fragments was isolated with KpnI (in the polylinker) and ClaI for microinjection.

cating that normal splicing occurred. To ascertain whether transcription was starting at the proper site and whether the same site was used for each of the constructs, we performed primer extension assays. Total RNA was hybridized with a ^{32}P-labeled oligonucleotide complementary to a sequence in the first exon of hGH mRNA and extended with reverse transcriptase. We expected an extension product of 72 nucleotides if the normal albumin cap site was being used. Indeed, as shown in Figure 3, a prominent extension product of

that length was observed with all samples tested, including liver samples from transgenic mice with the 0.3-, 0.9-, 3.8-, and 12-kb constructs and a kidney sample from a mouse with the 0.9-kb construct.

The enhancer-like element between −8.5 and −12 kb is sufficient for high-level hepatic expression

To ascertain whether there might be multiple enhancer-like elements in the 12 kb of albumin 5' flanking region,

Figure 2. hGH mRNA levels in liver and kidney of transgenic mice bearing the alb–hGH constructs shown in Fig. 1. hGH mRNA was measured by solution hybridization as described under Materials and methods. Each value corresponds to an individual founder transgenic mouse; values below the origin represent mice in which no hGH mRNA could be detected (less than 10 molecules/cell); solid symbols correspond to liver values of mice that grew more than 1.3-fold larger than control littermates; open symbols are used for those mice that did not grow larger than normal. Liver values are presented as circles, and kidney values as squares. Histograms represent the mean levels of liver hGH mRNA, based on only those mice that expressed the gene. Note the change in scale at 250 and 5000 molecules/cell.
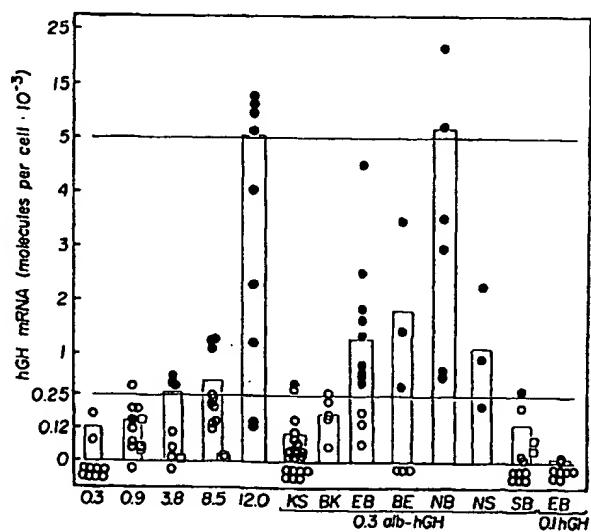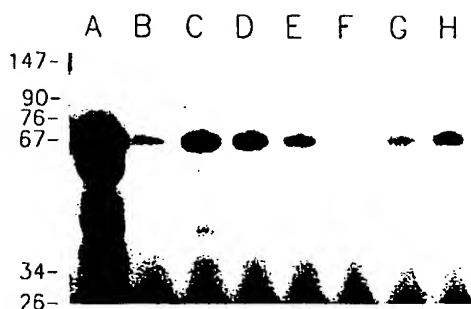
Figure 3. Primer extension analysis of alb–hGH transcripts. Total RNA was prepared, 50 μg was hybridized with an end-labeled oligonucleotide and extended with reverse transcriptase, and the extension products were displayed on an acrylamide gel as described under Materials and methods. Markers are end-labeled HpaII fragments from pBR322. (Lane A) Liver RNA from a mouse bearing a 12-kb alb–hGH construct (this RNA sample was from a mouse with 12,000 hGH mRNA molecules/cell; consequently, the lane is overexposed and reveals prominent bands of smaller size which probably represent premature termination products due to excess template); (lanes B and C) liver RNA from mice bearing a 3.8-kb alb–hGH construct; (lanes D and E) liver RNA from mice bearing the 0.9-kb alb–hGH construct; (lane F) kidney RNA from a mouse with 0.9-kb alb–hGH construct; (lanes G and H) liver RNA from mice with 0.3-kb alb–hGH construct. The EB 0.3 alb–hGH construct also gives a prominent band similar to that shown in lanes B–H (data not shown).

as is the case for the closely related and closely linked α-fetoprotein gene (Hammer et al. 1987), we tested three large regions for activity. We chose convenient restriction sites to generate fragments of about 3 kb each and positioned each fragment, in its normal orientation, directly upstream of the 0.3 alb–hGH construct. We tested these upstream fragments in the context of the albumin promoter region because previous data suggested that the albumin promoter has tissue-specific properties (see Introduction).

Only one of the three upstream fragments (the EcoRV–BamHI fragment; EB-0.3 alb–hGH) stimulated hepatic expression to levels higher than that observed with the 0.3 alb–hGH construct alone. Whereas only one of the 26 mice with either of the two proximal fragments grew larger than normal, 9 of 12 mice with the far-upstream EB fragment grew large and they had hepatic hGH mRNA levels that averaged about 1200 molecules per cell (Fig. 2). Moreover, no expression in kidney, or in any other tissue, was observed with this construct. Thus, the albumin sequence between −0.3 and −8.5 kb is unnecessary for enhanced levels of hepatic-specific expression in the adult.

The far-upstream albumin element functions in the reverse orientation but does not activate a heterologous promoter

One property of enhancers is that their orientation relative to a promoter can be reversed without affecting

their function (Serfling et al. 1985). To test this aspect of enhancer function, we reversed the orientation of the EcoRV–BamHI fragment upstream of the 0.3 alb–hGH construct. This reverse orientation (BE 0.3 alb–hGH) worked just as well as the normal orientation (Fig. 2).

Another property of many enhancers is that they will activate a heterologous promoter. We have shown that the immunoglobulin, elastase, or metallothionein enhancers, when placed 90 bp upstream of the hGH gene with its own promoter, can direct expression to appropriate tissues, whereas the enhancerless hGH gene is not expressed anywhere in transgenic mice (Hammer et al. and Ornitz et al., in prep.). Hence, we tested the EB fragment upstream of the enhancerless 0.1 hGH vector. Eight transgenic mice were produced with this construct, but none of them grew larger than normal and only two of them had a trace of hGH mRNA in the liver. Thus, it appears that the far-upstream albumin element does not function well with the hGH promoter and may require its own promoter.

More precise localization of the albumin enhancer-like elements

To localize the far-upstream element further, we tested various smaller fragments derived from the EB region, each upstream of the 0.3 alb–hGH construct. A NheI–BamHI fragment that encompassed the proximal half of the region (see Fig. 1) gave the highest average level of expression of all the constructs tested and all of the mice with this construct grew large (Fig. 2). This deletion narrowed the enhancer region down to about 1.9 kb. We then cut this region in half with ScaI and tested the distal NS fragment and the proximal SB fragment in the same manner. Four of 10 mice with the proximal SB region gave a low level of expression, comparable to that observed with 0.3 alb–hGH alone, and two of them expressed a low level of hGH mRNA in kidney as well. All three of the mice with the distal NS region had hepatic hGH mRNA levels averaging 10-fold higher than that observed with the promoter alone (Fig. 2). However, low expression (less than 20 molecules/cell) was also observed in tissues such as pancreas, intestine, and lung that had never been positive with other constructs. Thus, it appears that the NheI–ScaI region still has enhancer-like activity, but that elements within the adjacent region also play a role in determining hepatic-specific expression.

One of four DNase hypersensitive sites is located within the upstream enhancer region

The choice of restriction sites for the previous dissection of the upstream region was guided in part by the results of a search for DNase-sensitive sites in the albumin 5′-flanking region. Such hypersensitive sites may reflect the binding of regulatory proteins to specific DNA sequences (Elgin 1981). Two unique probes isolated from the albumin-flanking region, one centered at about −4 kb (probe 2) and the other centered at about −11 kb (probe 1), were used to map DNase hypersensitive re-

gions of the endogenous albumin gene. Liver nuclei were isolated and incubated at 25°C for various times to allow an endogenous DNase to make preferential cuts. Then the DNA was isolated, restricted with StuI or EcoRI to map 5' and 3' of probe 2, respectively, or with EcoRV to map 3' of probe 1 (see Fig. 4). Four DNase hypersensitive sites were observed: a prominent one (labeled HS1) is located at about −10.5 kb within the NheI–ScaI region


MOUSE ALBUMIN GENE NUCLEASE SENSITIVE SITES
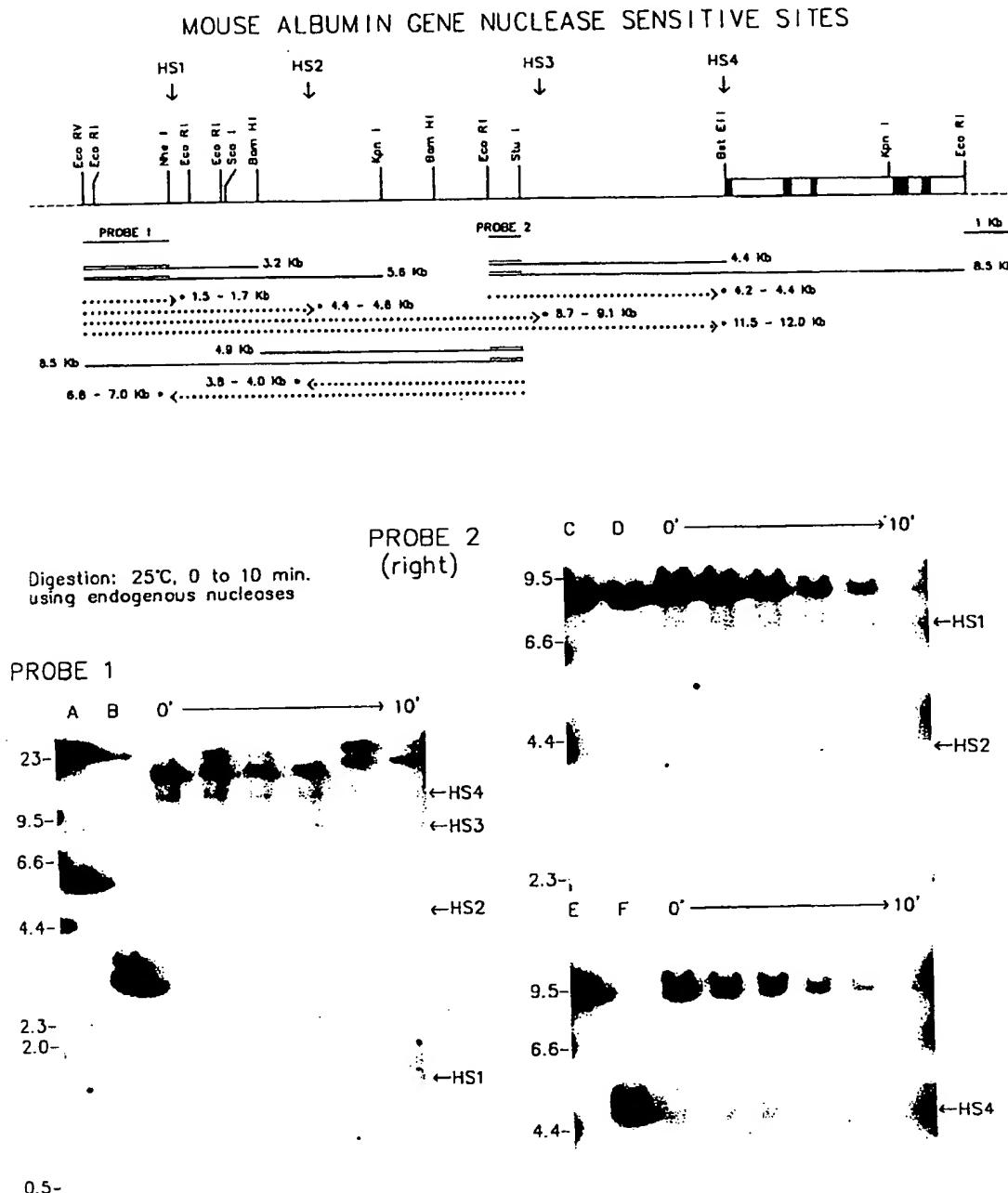
Figure 4. (See facing page for legend.)

that has enhancer-like activity, two less intense sites (HS2 and HS3) are located at about −7.5 and −3.1 kb, and a fourth intense site (HS4) is located in the vicinity of the promoter. Partial digestion by the endogenous nucleases during nuclei isolation probably accounts for the presence of most of these hypersensitive sites in the zero-time controls. Note, however, that HS1 and HS4 are absent in kidney nuclei (lanes C, D, and E).

Discussion

The 12 kb of mouse albumin 5'-flanking region appears to contain all the cis-acting elements necessary for appropriate expression of the albumin gene in the adult. Based on the analysis presented here, two regions are critical for liver-specific expression in the adult. One is the 300-bp promoter region, which had been shown previously to be a tissue-specific promoter (see Introduction), and the other is an enhancer-like element, or elements, that lie within the NheI–BamHI region, 8.5–10.4 kb upstream of the promoter. When these two regions are juxtaposed, as in the NB 0.3 alb–hGH construct, they stimulate the production of hGH mRNA levels comparable to endogenous albumin mRNA (see Fig. 2). Although the high level of expression achieved with this construct suggests that all the essential cis-acting elements are present within the 12 kb of albumin 5'-flanking region, we cannot rule out the possibility that there may be other elements either further upstream, within, or downstream of the albumin gene that also contribute to appropriate expression. This uncertainty stems from the comparison of hGH and albumin mRNA levels, which may have different half-lives, and the uncertainty of knowing how many transgenes contribute to hGH mRNA accumulation. All of the transgenic mice studied in this report were founder animals, hence some of them were surely mosaics with the transgene in only a fraction of their cells (Wilkie et al. 1986). Moreover, most of the mice have multiple copies of the transgene and, like most other constructs tested in mice, there is little relationship between gene copy number and the level of expression; thus, we cannot know how many of the transgene copies are actually contributing to expression. Nevertheless, these results indicate that important control elements can lie great distances away

from the structural genes that they regulate. Because the distance is about 10 kb, we favor the idea that this region is brought into proximity to the promoter by DNA looping rather than by some propagation of information along the DNA from the upstream element to the promoter (Serfling et al. 1985; Ptashne 1986).

The far-upstream albumin region has some of the properties of an enhancer, in that its position and orientation relative to the promoter do not seem to be critical. However, it failed to promote significant levels of expression when combined with the heterologous hGH promoter, which has responded to several other enhancers. Although it is possible that the albumin enhancer might function in conjunction with other promoters, it is also possible that it can only function in conjunction with its own or other liver-specific promoters. Thus, at present, we can only say that it enhances the function of the albumin promoter about 50-fold. It is curious that the albumin sequences between −8.5 and −12 kb also appear to suppress the kidney expression observed in some transgenic mice bearing constructs that contain the region between −0.3 and −0.9 kb.

The smallest, most effective albumin region tested thus far is about 1.9 kb. Although other truncated versions remain to be tested, it seems likely that the albumin element may not be as compact as other enhancer elements. This conclusion is based on the observation that the 1.9-kb NheI–BamHI region is very effective whereas the smaller NheI–ScaI region is less effective quantitatively and allows expression in other tissues. Furthermore, the neighboring ScaI–BamHI fragment does not stimulate hepatic expression but it does promote nonhepatic expression.

In the process of dissecting three different enhancers, we have noted that tissue specificity is lost before enhancing ability is lost. In addition to the example above, when the elastase element was trimmed from 134 bp to 80 bp it allowed promiscuous expression (Hammer et al., in prep.) and when a DraI–PstI fragment that includes the core of the immunoglobulin heavy-chain enhancer was tested, it allowed expression in several tissues in addition to B cells (Ornitz et al., in prep.). These observations suggest that each of these enhancers may contain core elements that bind general enhancing factors and that enhancing activity is restricted to specific cell

Figure 4.   DNase hypersensitive sites in albumin 5'-flanking region. (Top) Map of the albumin gene extending from −12 kb to +4 kb (in the 5th intron). Albumin exons are solid boxes, introns are open boxes. Arrows labeled HS1 to HS4 indicate the positions of the hypersensitive sites mapped as described below. Probe 1 is a unique EcoRV–NheI fragment; probe 2 is a unique EcoRI–StuI fragment. Genomic restriction fragments used as internal size markers are shown as solid lines paired with the appropriate probes. Dotted lines represent the size of the bands mapped with probes 1 and 2 and visualized on the autoradiographs shown below. (Left) Probe 1 was used to map nuclease-sensitive sites downstream of EcoRV. (Lane A) DNA from liver nuclei restricted with EcoRV and KpnI; (lane B) DNA from liver nuclei restricted with EcoRV and BamHI; (lanes 0'–10') digestion products from liver nuclei treated for 0–10 min at 25°C and then restricted with EcoRV. (Center right) Probe 2 was used to map nuclease-sensitive sites upstream of the StuI site. (Lane C) DNA from kidney nuclei restricted with StuI; (lane D) DNA from kidney nuclei restricted with StuI and EcoRV; (lanes 0'–10') liver nuclei treated for 0–10 min at 25°C and then restricted with StuI. (Bottom right) Probe 2 was used to map hypersensitive sites downstream of EcoRI. (Lane E) DNA from kidney nuclei restricted with EcoRI; (lane F) DNA from kidney nuclei restricted with EcoRI and BstEII; (lanes 0'–10') liver nuclei treated at 25°C for 0–10 min and then digested with EcoRI. Markers are λ DNA restricted with HindIII.

types by factors that bind to adjacent regions. In some cases, all of the binding sites may be arranged in a very compact region, as in the case of the elastase enhancer, whereas in other cases the binding sites may be dispersed over several hundred base pairs as in albumin and immunoglobulin genes.

The binding of proteins to the albumin promoter and the far-upstream albumin region is consistent with the presence of DNase hypersensitive sites in these regions (Fig. 4). We noted two other hypersensitive regions between the promoter and the far-upstream regions, although we cannot ascribe any function to them. They might be involved in developmental or environmental control of albumin gene expression. Babiss et al. (1986) also noted DNase hypersensitive sites in the rat albumin gene located near the promoter and about 2.8 kb upstream; they did not look more then 7 kb upstream.

At this level of analysis, it appears that the organization of the regulatory elements for albumin and α-fetoprotein genes is dissimilar. The only similarity is that both promoters appear to be tissue specific, although neither functions very well in transgenic mice in the absence of upstream enhancer elements (Hammer et al. 1987). Whereas the enhancer elements for the α-fetoprotein gene are spread out in at least three regions spanning about 6 kb (Hammer et al. 1987), the albumin enhancer lies in a 1.9-kb region positioned nearly 10 kb upstream of the gene. However, it is important to recall that we are comparing the sequences that are important for expression of the albumin gene in the adult and expression of the α-fetoprotein gene in the developing fetus. Perhaps the sequences responsible for fetal expression of the albumin gene are the same ones that are used for α-fetoprotein gene expression; alternatively, they might be duplicated in the region between −0.3 and −8.5 kb. Thus, it is conceivable that the elements involved in the fetal expression of the two genes are similar and the far-upstream albumin elements are critical for maintenance of expression in adult liver. Ultimately, it will be informative to compare the DNA sequences of the enhancers for these two genes and the proteins that interact with them.

We originally planned to generate lines of transgenic mice that express alb-hGH fusion genes so that offspring could be used to study developmental and environmental control of transgene expression. However, the use of hGH as the reporter gene has precluded the generation of any lines that express significant amounts of hGH because the reproductive performance of both transgenic males and females was impaired. We anticipated some problem with females, since transgenic females that express metallothionein–hGH fusion genes are relatively infertile (Hammer et al. 1985), but the problem seems to be more severe with the alb-hGH constructs and both sexes are affected. The liver is also a prime site of synthesis of hGH when the metallothionein promoter is used; nevertheless, MT–hGH lines have been established and maintained for many generations (R. Hammer and R. Brinster, unpubl.). Perhaps some critical differences in the expression of these two

transgenes during fetal development affects reproductive function.

These results reveal how the interpretation of results from enhancer studies depends on the reporter gene and the frame of reference. For example, albumin–CAT constructs have been instructive in terms of demonstrating that the albumin promoter functions better when transfected into hepatocytes than when tested in a few other cell types (Ott et al. 1984). However, because CAT enzyme activity was measured, it was not possible to know whether the level of albumin–CAT expression was quantitatively appropriate. Moreover, most hepatocyte cell lines do not make normal amounts of albumin mRNA, perhaps due to depletion of active enhancer-binding factors; thus, these cell lines may be unsuitable for detecting enhancer function. In conclusion, one cannot feel confident that all of the cis-acting elements have been included in a construct unless expression is developmentally appropriate, cell-specific, and quantitatively normal. The last parameter is difficult to measure and can only be satisfactorily answered by using a reporter gene that produces a mRNA with the same half-life as the endogenous gene or by measuring transcription rates. In both cases, one would ideally choose animals or cells in which only one gene was integrated so there would be no ambiguity about how many transgenes were functional.

The 12-kb albumin 5'-flanking region has been used effectively to direct the expression of other structural genes to the liver. For example, it will direct hepatitis B surface antigen (HBsAg) to hepatic cells, as visualized by immunofluorescence (Chisari et al. 1986). It has also been used to direct the expression of SV40 T-antigen and human c-H-ras genes to liver (E. Sandgren et al., unpubl.). Expression of each of these gene products leads to characteristic liver pathology. Expression of the hepatitis gene leads to accumulation of hepatitis large-envelope polypeptide in the endoplasmic reticulum and this is associated with progressive liver cell injury (Chisari et al. 1986 and unpubl.), whereas expression of either T antigen or an activated form of c-H-ras leads to hepatocellular tumors (E. Sandgren et al., unpubl.). Even the expression of hGH in the liver appears to be deleterious in that characteristic dysplasia is evident by 3 months, and by 7 months there is an obvious disorganization of normal lobular architecture (C. Pinkert et al., unpubl.). These examples serve to illustrate that the albumin promoter/enhancer can be used to generate animal models of human diseases in which the effects of various gene products on hepatic function can be studied.

Materials and methods

Construction of alb–hGH fusion genes and production of transgenic mice

A mouse albumin subclone spanning the promoter region was digested with BstEII, which cut at a unique site about 50 bp downstream of the cap site. The DNA was digested with ExoIII for varying times, treated with S1 nuclease and Klenow fragment of DNA polymerase to produce blunt ends, and then li-

gated with phosphorylated BamHI linkers. A clone with a BamHI linker positioned between the cap site and initiation codon was identified by acrylamide gel electrophoresis and the exact position was determined by DNA sequencing. The albumin promoter and about 4 kb of 5'-flanking region was fused to the hGH structural gene at its unique BamHI site located at +3. A unique SaII linker was introduced beyond the polyadenylation site of hGH, then the albumin 5' region was extended to 12 kb by inserting a StuI fragment isolated from a λ clone carrying albumin 5'-flanking sequences. Unique EcoRV and BssHII sites lie at about −12 kb so that the entire alb–hGH region can be isolated with either of these enzymes and SaII. To construct internal deletions, the alb–hGH gene was digested with SphI, which cuts at −0.3 kb in the albumin gene and beyond the polyadenylation site of hGH, and this piece was cloned into the SphI site of pUC18 to create a vector with a polylinker upstream of the albumin promoter. The 3' SphI site was converted to a unique ClaI site and then various 5' albumin fragments were cloned into the polylinker (Fig. 1). An NsiI site located at −90 in the hGH gene was used for the construction of the EB-0.1–hGH construct.

DNA fragments for microinjection were separated from remaining plasmid DNA by agarose gel electrophoresis, isolated by dissolving the gel in NaClO₄ and binding the DNA to glass (Whatman GF/C filters), and eluting with 1 mM Tris-HCl. The DNA concentration was measured and diluted to 2 ng/μl for microinjection. About 2 pl of the DNA solution was microinjected into pronuclei of fertilized C57/SJL F₂ hybrid mouse eggs. Eggs that survived injection were transferred to pseudopregnant recipients for continued development. Transgenic pups were identified by dot analysis of tail nucleic acids using a nick-translated hGH probe (Brinster et al. 1985).

Analysis of hGH mRNA levels

Total nucleic acids were isolated by homogenizing 50–100 mg of tissue in 4 ml of SET buffer with 100 μ/ml of proteinase K, incubating the extract at room temperature for 24 hr, and then isolating total nucleic acids by phenol/chloroform extraction and ethanol precipitation (Durnam and Palmiter 1983). hGH mRNA levels were determined by solution hybridization using a 21-base oligonucleotide complementary to a sequence in the fourth exon of hGH and M13 single-stranded DNA as a standard (Ornitz et al. 1985a). This assay is sensitive enough to measure 10 molecules of hGH/cell.

For primer extension, an oligonucleotide, GTGGACAGCT-CACCTAGCTAGCTGCTGCAAT, complementary to a sequence in the first exon of hGH mRNA was end-labeled with ³²P using T4 kinase, hybridized to 50 μg RNA prepared from total nucleic acids by treatment of DNase I, and then extended with AMV reverse transcriptase. The extension products were analyzed on a denaturing acrylamide gel (Townes et al. 1985).

DNase hypersensitivity assays

Liver nuclei were isolated as described by Ornitz et al. (1985b) and incubated at 25°C for up to 10 min to allow endogenous DNases to nick the DNA; then DNA was isolated by treatment with SDS and proteinase K followed by phenol/chloroform extraction and ethanol precipitation. DNA was digested with an appropriate restriction enzyme, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated probe (Ornitz et al. 1985).

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ProductInformation

MCDB 153 MEDIUM
With L-Glutamine and 28 mM HEPES,
Without Sodium Bicarbonate

Product Number M 7403
Storage Temperature 2-8 °C

Product Description

MCDB media were designed for the low-protein or serum-free growth of specific cell types using hormones, growth factors, trace elements or low levels of dialyzed fetal bovine serum protein (FBSP). Each MCDB medium was formulated (qualitatively and quantitatively) to provide a defined and optimally balanced nutritional environment that selectively promoted growth of a specific cell type. MCDB 105 and 110 are modifications of MCDB 104 medium, optimized for long-term survival and rapid clonal growth of human diploid fibroblast-like cells (WI-38, MRC-5, IMR-90) and of low-passage human foreskin fibroblasts using FBSP or hormone and growth factor supplements. MCDB 151, 153, 201 and 302 are modifications of Ham's nutrient mixture F-12, designed for the growth of human keratinocytes, clonal growth of chicken embryo fibroblasts and chinese hamster ovary (CHO) cells using low levels of FBSP, extensive trace elements or no serum protein.

MCDB 153 MEDIUM, Product No. M 7403 is one of the cell culture media available from Sigma. The selection of a nutrient medium is strongly influenced by 1] type of cell, 2] type of culture [monolayer, suspension, clonal] and 3] degree of chemical definition necessary. It is important to review the literature for recommendations concerning medium, supplementation and physiological parameters required for a specific cell line.

Components	g/L
Ammonium Metavanadate	0.000000585
Calcium Chloride·Anhydrous	0.00333
Cupric Sulfate·5H$_2$O	0.00000275
Ferrous Sulfate·7H$_2$O	0.00139
Magnesium Chloride	0.05713
Manganese Sulfate	0.000000151
Molybdic Acid·4H$_2$O (ammonium)	0.00000124
Nickel Chloride·6H$_2$O	0.00000012
Potassium Chloride	0.11183
Sodium Acetate (anhydrous)	0.30153
Sodium Chloride	7.599
Sodium Metasilicate·9H$_2$O	0.000142
Sodium Phosphate Dibasic (anhydrous)	0.284088
Sodium Selenite	0.0000038
Stannous Chloride·2H$_2$O	0.000000113
Zinc Sulfate·7H$_2$O	0.000144
L-Alanine	0.00891
L-Arginine·HCl	0.2107
L-Asparagine·H$_2$O	0.015
L-Aspartic Acid	0.00399
L-Cysteine·HCl·H$_2$O	0.04204
L-Glutamic Acid	0.01471
L-Glutamine	0.8772
Glycine	0.00751
L-Histidine·HCl·H$_2$O	0.01677
L-Isoleucine	0.001968
L-Leucine	0.0656
L-Lysine·HCl	0.01827
L-Methionine	0.00448
L-Phenylalanine	0.00496
L-Proline	0.03453
L-Serine	0.06306
L-Threonine	0.01191
L-Tryptophan	0.00306
L-Tyrosine·2Na	0.00341
L-Valine	0.03513
D-Biotin	0.0000146
Choline Chloride	0.01396
Folic Acid	0.00079
myo-Inositol	0.01802
Niacinamide	0.00003663
D-Pantothenic Acid (hemicalcium)	0.000238
Pyridoxine·HCl	0.00006171
Riboflavin	0.0000376
Thiamine·HCl	0.000337

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Vitamin B-12	0.000407
Adenine·HCl	0.03088
D-Glucose	1.081
HEPES	6.6
Phenol Red·Na	0.001242
Putrescine·2HCl	0.000161
Pyruvic Acid·Na	0.055
Thioctic Acid	0.000206
Thymidine	0.000727

Precautions and Disclaimer
For R&D use only.
Not for drug, household or other uses.

Preparation Instructions
Powdered media are extremely hygroscopic and should be protected from atmospheric moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form.

Supplements can be added prior to filtration or introduced aseptically to sterile medium. The nature of the supplement may affect storage conditions and shelf life of the medium.

1. Measure out 90% of final required volume of water. Water temperature should be 15-20 °C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 1.2 g sodium bicarbonate or 15.7 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.

Storage/Stability
Store the dry powdered medium at 2-8 °C under dry conditions and liquid medium at 2-8 °C in the dark. Deterioration of the powdered medium may be recognized by any or all of the following: [1] color change, [2] granulation/clumping, [3] insolubility. Deterioration of the liquid medium may be recognized by any or all of the following: [1] pH change, [2] precipitate or particulate matter throughout the solution, [3] cloudy appearance [4] color change. The nature of supplements added may affect storage conditions and shelf life of the medium. Product label bears expiration date.

Procedure
MATERIALS REQUIRED BUT NOT PROVIDED:
Water for tissue culture use [W 3500]
Sodium Bicarbonate [S 5761] or
Sodium Bicarbonate Solution, 7.5% [S 8761]
1N Hydrochloric Acid [H 9892]
1N Sodium Hydroxide [S 2770]
Medium additives as required

Product Profile

Appearance	off-white powder
Moisture content	≤2.0%
Solubility	clear solution at 1x concentration
pH at room temperature [without sodium bicarbonate]	6.1 ± 0.3
pH at room temperature [with sodium bicarbonate]	6.7 ± 0.3
Osmolality [without sodium bicarbonate]	293 mOsm/kg H_2O ± 5%
Osmolality [with sodium bicarbonate]	322 mOsm/kg H_2O ± 5%
Endotoxin	≤1.0 EU/ml at 1x
Amino Acid Analysis by HPLC	Analysis has confirmed that amino acids are present at concentrations consistent with the formula.
Key Element Analysis by ICAP	Analysis has confirmed that key elements are present at concentrations consistent with the formula.

BIOLOGICAL PERFORMANCE CHARACTERISTICS

Biological performance is assessed using an appropriate cell line(s). Growth studies are carried through 2 subculture generations. Cells are counted and growth is plotted as a logarithmic function of time in culture. Seeding efficiencies, doubling time, and final cell densities are determined. During the testing period cultures are examined microscopically for atypical morphology and evidence of cytotoxicity. Test results are available upon request.

References

1. Boyce, S.T. and Ham, R.G., (1983). Calcium-Regulated Differentiation of Normal Human Epidermal Keratinocytes in Chemically Defined Clonal Culture and Serum-Free Serial Culture. J. Invest. Dermatol, 81, 33-40.
2. McKeehan, W.L. and Ham, R.G., (1976). Stimulation of Clonal Growth of Normal Fibroblasts with Substrata Coated with Basic Polymers. J. Cell Biol., 71, 727-734.
3. Hamilton, W.G. and Ham, R.G., (1977). Clonal Growth of Chinese Hamster Ovary Cell Lines in Protein-Free Media. In Vitro, 13:9, 537-547.

Revision date: 2003-12

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SIGMA

ProductInformation

Nutrient Mixtures (Ham)

Ham's Nutrient Mixtures were originally developed to support clonal growth of several clones of Chinese hamster ovary (CHO) cells, as well as clones of HeLa and mouse L-cells. Both mixtures were formulated for use with or without serum supplementation, depending on the cell type being cultured. Ham's F-10 has been shown to support the growth of human diploid cells, white blood cells for chromosomal analysis, primary explants of rat, rabbit and chicken tissues. Ham's F-12 has been used for the growth of primary rat hepatocytes and rat prostate epithelial cells. A clonal toxicity assay using CHO cells has also been reported with Ham's F-12 as the medium of choice. Ham's F-12 is also available with 25mM HEPES buffer that provides more effective buffering in the optimum pH range of 7.2-7.4.

COMPONENT	N 6635 (F-10) g/L	N 1387 (F-10) g/L	N 6760 (F-12) g/L	N 4388 (F-12) g/L
INORGANIC SALTS				
$CaCl_2 \cdot 2H_2O$	0.0441	0.0441	0.0441	0.0441
$CuSO_4 \cdot 5H_2O$	0.0000025	0.0000025	0.0000025	0.0000025
$FeSO_4 \cdot 7H_2O$	0.000834	0.000834	0.000834	0.000834
$MgCl \cdot 6H_2O$	—	—	0.123	0.123
$MgSO_4$	0.07464	0.07464	—	—
KCl	0.285	0.285	0.224	0.224
KH_2PO_4	0.083	0.083	—	—
NaCl	7.4	6.8	7.599	7.1
Na_2HPO_4	0.1537	0.1537	0.14204	0.14204
$ZnSO_4 \cdot 7H_2O$	0.0000288	0.0000288	0.000863	0.000863
AMINO ACIDS				
L-Alanine	0.009	0.009	0.009	0.009
L-Arginine·HCl	0.211	0.211	0.211	0.211
L-Asparagine·H₂O	0.01501	0.01501	0.01501	0.01501
L-Aspartic Acid	0.0133	0.0133	0.0133	0.0133
L-Cysteine·HCl·H₂O	0.035	0.035	0.035	0.035
L-Glutamic Acid	0.0147	0.0147	0.0147	0.0147
L-Glutamine	0.146	0.146	0.146	0.146
Glycine	0.00751	0.00751	0.00751	0.00751
L-Histidine·HCl·H₂O	0.021	0.021	0.02096	0.02096
L-Isoleucine	0.0026	0.0026	0.00394	0.00394
L-Leucine	0.0131	0.0131	0.0131	0.0131
L-Lysine·HCl	0.0293	0.0293	0.0365	0.0365
L-Methionine	0.00448	0.00448	0.00448	0.00448
L-Phenylalanine	0.00496	0.00496	0.00496	0.00496
L-Proline	0.0115	0.0115	0.0345	0.0345
L-Serine	0.0105	0.0105	0.0105	0.0105
L-Threonine	0.00357	0.00357	0.0119	0.0119
L-Tryptophan	0.0006	0.0006	0.00204	0.00204
L-Tyrosine 2Na·2H₂O	0.00261	0.00261	0.00778	0.00778
L-Valine	0.0035	0.0035	0.0117	0.0117
VITAMINS				
D-Biotin	0.000024	0.000024	0.0000073	0.0000073
Choline Chloride	0.000698	0.000698	0.01396	0.01396
Folic Acid	0.00132	0.00132	0.00132	0.00132
myo-Inositol	0.000541	0.000541	0.018	0.018
Niacinamide	0.000615	0.000615	0.000037	0.000037
D-Pantothenic Acid ·½Ca	0.000715	0.000715	0.00048	0.00048
Pyridoxine·HCl	0.000206	0.000206	0.000062	0.000062
Riboflavin	0.000376	0.000376	0.000038	0.000038
Thiamine·HCl	0.001	0.001	0.00034	0.00034
Vitamin B-12	0.00136	0.00136	0.00136	0.00136

Formulas continued next page

Nutrient Mixtures (Ham) continued

COMPONENT	N 6635 (F-10) g/L	N 1387 (F-10) g/L	N 6760 (F-12) g/L	N 4388 (F-12) g/L
OTHER				
D-Glucose	1.1	1.1	1.802	1.802
HEPES	—	5.958	—	5.958
Hypoxanthine	0.00408	0.00408	0.00408	0.00408
Linoleic Acid	—	—	0.000084	0.000084
Phenol Red·Na	0.0013	0.0013	0.0013	0.0013
Putrescine·HCl	—	—	0.000161	0.000161
Pyruvic Acid·Na	0.11	0.11	0.11	0.11
Thioctic Acid	0.00021	0.00021	0.00021	0.00021
Thymidine	0.00073	0.00073	0.00073	0.00073
ADD				
Sodium Bicarbonate	1.2	1.2	1.176	1.176
Grams of powder required to prepare 1 L	9.8	15.2	10.7	16.2

Formulas continued next page

Nutrient Mixtures (Ham) continued

COMPONENT	N 6013 (F-10) [1X] g/L	N 2147 (F-10) [1X] g/L	N 4888 (F-12) [1X] g/L	N 8641 (F-12) [1X] g/L
INORGANIC SALTS				
$CaCl_2 \cdot 2H_2O$	0.0441	0.0441	0.0441	0.0441
$CuSO_4 \cdot 5H_2O$	0.0000025	0.0000025	0.0000025	0.0000025
$FeSO_4 \cdot 7H_2O$	0.000834	0.000834	0.000834	0.000834
$MgCl \cdot 6H_2O$	—	—	0.123	0.123
$MgSO_4$	0.07464	0.07464	—	—
KCl	0.285	0.285	0.224	0.224
KH_2PO_4	0.083	0.083	—	—
$NaHCO_3$	1.2	—	1.176	1.176
NaCl	7.4	7.4	7.599	7.1
Na_2HPO_4	0.1537	0.1537	0.14204	0.14204
$ZnSO_4 \cdot 7H_2O$	0.0000288	0.0000288	0.000863	0.000863
AMINO ACIDS				
L-Alanine	0.009	0.009	0.009	0.009
L-Arginine·HCl	0.211	0.211	0.211	0.211
L-Asparagine·H_2O	0.01501	0.01501	0.01501	0.01501
L-Aspartic Acid	0.0133	0.0133	0.0133	0.0133
L-Cysteine·HCl·H_2O	0.035	0.035	0.035	0.035
L-Glutamic Acid	0.0147	0.0147	0.0147	0.0147
Glycine	0.00751	0.00751	0.00751	0.00751
L-Histidine·3HCl·H_2O	0.021	0.021	0.02096	0.02096
L-Isoleucine	0.0026	0.0026	0.00394	0.00394
L-Leucine	0.0131	0.0131	0.0131	0.0131
L-Lysine·HCl	0.0293	0.093	0.0365	0.0365
L-Methionine	0.00448	0.00448	0.00448	0.00448
L-Phenylalanine	0.00496	0.00496	0.00496	0.00496
L-Proline	0.0115	0.0115	0.0345	0.0345
L-Serine	0.0105	0.0105	0.0105	0.0105
L-Threonine	0.00357	0.00357	0.0119	0.0119
L-Tryptophan	0.0006	0.0006	0.00204	0.00204
L-Tyrosine 2Na·$2H_2O$	0.00261	0.00261	0.00778	0.00778
L-Valine	0.0035	0.0035	0.0117	0.0117
VITAMINS				
D-Biotin	0.000024	0.000024	0.0000073	0.0000073
Choline Chloride	0.000698	0.000698	0.01396	0.01396
Folic Acid	0.00132	0.00132	0.00132	0.00132
myo-Inositol	0.000541	0.000541	0.018	0.018
Niacinamide	0.000615	0.000615	0.000037	0.000037
D-Pantothenic Acid ·½Ca	0.000715	0.000715	0.00048	0.000238
Pyridoxine·HCl	0.000206	0.000206	0.000062	0.000062
Riboflavin	0.000376	0.000376	0.000038	0.000038
Thiamine·HCl	0.001	0.001	0.00034	0.00034
Vitamin B-12	0.00136	0.00136	0.00136	0.00136
OTHER				
D-Glucose	1.1	1.1	1.802	1.802
HEPES	—	4.77	—	5.958
Hypoxanthine	0.00408	0.00408	0.00408	0.00408
Linoleic Acid	—	—	0.000084	0.000084
Phenol Red (sodium)	0.0013	0.0013	0.0013	0.0013
Putrescine·HCl	—	—	0.000161	0.000161
Pyruvic Acid (sodium)	0.11	0.11	0.11	0.11
Thioctic Acid	0.00021	0.00021	0.00021	0.00021
Thymidine	0.00073	0.00073	0.00073	0.00073

Formulas continued next page

Nutrient Mixtures (Ham) continued

COMPONENT	N 6013 (F-10) [1X] g/L	N 2147 (F-10) [1X] g/L	N 4888 (F-12) [1X] g/L	N 8641 (F-12) [1X] g/L
ADD				
L-Glutamine	0.146	0.146	0.146	0.146
Sodium Bicarbonate	—	—	—	—

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1. Ham, R.G., (1963). An Improved Nutrient Solution for Diploid Chinese Hamster and Human Cell Lines. Exp. Cell Res. 29, 515-526.
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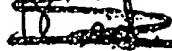
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Modulation of murine melanocyte function *in vitro* by agouti signal protein

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[6]Corresponding author

Molecular and biochemical mechanisms that switch melanocytes between the production of eumelanin or pheomelanin involve the opposing action of two inter-cellular signaling molecules, α-melanocyte-stimulating hormone (MSH) and agouti signal protein (ASP). In this study, we have characterized the physiological effects of ASP on eumelanogenic melanocytes in culture. Following exposure of black melan-a murine melanocytes to purified recombinant ASP *in vitro*, pigmentation was markedly inhibited and the production of eumelanosomes was decreased significantly. Melanosomes that were produced became pheomelanosome-like in structure, and chemical analysis showed that eumelanin production was significantly decreased. Melanocytes treated with ASP also exhibited time- and dose-dependent decreases in melanogenic gene expression, including those encoding tyrosinase and tyrosinase-related proteins 1 and 2. Conversely, melanocytes exposed to MSH exhibited an increase in tyrosinase gene expression and function. Simultaneous addition of ASP and MSH at approximately equimolar concentrations produced responses similar to those elicited by the hormone alone. These results demonstrate that eumelanogenic melanocytes can be induced in culture by ASP to exhibit features characteristic of pheomelanogenesis *in vivo*. Our data are consistent with the hypothesis that the effects of ASP on melanocytes are not mediated solely by inhibition of MSH binding to its receptor, and provide a cell culture model to identify novel factors whose presence is required for pheomelanogenesis.

Keywords: agouti/melanogenesis/pheomelanin/pigmentation/tyrosinase

Introduction

The *agouti* (a) locus is one of >60 distinct genes that regulate coat color in mice (Silvers, 1979). The product of that locus (termed agouti signal protein, ASP) is produced by dermal papillae cells (Millar *et al.*, 1995) and is a paracrine factor that modulates the production of pigment by follicular melanocytes (Bultman *et al.*, 1992; Miller *et al.*, 1993). More specifically, it controls whether black/brown eumelanin or yellow/red pheomelanin is produced, although the mechanism by which this switch is effected is as yet unknown. In mice that carry the A allele, eumelanin is produced by all follicular melanocytes at the beginning of the hair growth cycle (i.e. from 0 to 4 days). Transient expression of ASP from 4 to 6 days of the hair cycle causes melanocytes to produce pheomelanin instead of eumelanin; after 6 days, *agouti* gene expression is turned off and eumelanin is produced again. This pattern of pigment synthesis results in a yellow striped band near the tip of each hair shaft against a black background. Eumelanin and pheomelanin differ not only in their gross appearance, but also in their chemical composition and the ultrastructure of the melanosomes in which they are synthesized and deposited. Follicular melanocytes of 1- to 2-day-old agouti mice contain ellipsoid and fibrillar eumelanosomes, while follicular melanocytes of 4- to 6-day-old agouti mice contain ovoid and particulate pheomelanosomes. Similar changes in pheomelanosomes and eumelanosomes have been confirmed in a number of mouse mutants which produce one or the other type of melanin (Prota *et al.*, 1995).

Mutations at the *agouti* locus can cause the production of all yellow or all black hair, depending on whether the mutation leads to overexpression/hyperfunction or non-expression/non-function of ASP, respectively (Perry *et al.*, 1994; Siracusa, 1994; Vrieling *et al.*, 1994). As examples, the dominant lethal yellow mutation (A^y) results in the production of completely yellow hairs (Miller *et al.*, 1993; Duhl *et al.*, 1994; Michaud *et al.*, 1994), while the recessive non-agouti (a) (Bultman *et al.*, 1994), lethal non-agouti (a^x) (Miller *et al.*, 1994) and extreme non-agouti (a^e) (Hustad *et al.*, 1995) alleles cause the production of completely black hairs. Consequently, the *agouti* locus has been recognized to have an important role in regulating the switch between the production of eumelanin or pheomelanin by melanocytes.

The biochemical action of ASP is controversial and has been the source of continuing debate (Conklin and Bourne, 1993; Jackson, 1993; Yen *et al.*, 1994). Several studies (Lu *et al.*, 1994; Blanchard *et al.*, 1995; Willard *et al.*, 1995; Siegrist *et al.*, 1996) have shown that ASP antagonizes the action of α-melanocyte-stimulating hormone (MSH) in activating the melanocyte-specific MSH receptor (MC1-R), which suggests that the effect of the *agouti* locus on melanocytes is mediated by reduced signaling through the MC1-R, while its extrapigmentary effects may be mediated by reduced signaling through other melanocortin receptors. By contrast, the similarity of ASP

to the conotoxins and its ability to elevate intracellular calcium has also been noted (Manne et al., 1995; Willard et al., 1995; Zemel et al., 1995; Perry et al., 1996), suggesting that some effects of ASP might be mediated by an alteration in calcium channels, a mechanism supported by Hunt and Thody (1995) who found that ASP antagonizes the stimulation of melanogenesis by verapamil (a calcium modulator).

In vivo, we have reported (Kobayashi et al., 1995) that the expression and enzyme activity of tyrosinase was reduced in follicular melanocytes of lethal yellow mice and of 5- to 7-day-old agouti mice but that there was little or no expression or enzyme activity of tyrosinase-related proteins 1 (TRP1) and 2 (TRP2) during pheomelanogenesis. This pattern of expression is consistent with the fact that tyrosinase is required for both types of pigment synthesis, but expression of the two tyrosinase-related proteins (TRP1 and TRP2) is required only for eumelanin synthesis (Tsukamoto et al., 1992; Kobayashi et al., 1994; Winder et al., 1994).

We now describe studies examining the effect(s) of purified recombinant ASP on cultured melanocytes. Following treatment with ASP, eumelanogenic melanocytes in culture exhibit physiologic features characteristic of pheomelanogenesis in vivo, thus providing an in vitro model for characterization of the mechanisms and genes involved in this switch.

Results

mRNA levels of melanogenic genes following treatment with ASP

We initially examined whether ASP had any effect on steady-state mRNA levels of melanogenic genes using Northern blotting of melan-a melanocytes treated with varying concentrations of ASP for 24 h (Figure 1). There were significant dose-dependent decreases in the expression of tyrosinase, TRP1 and TRP2 mRNAs following treatment with ASP at 10 and 1 nM. However, there was no significant effect on the level of mRNA for MC1-R at any ASP concentration tested. The numbers reported below the bands in each of the figures represent the quantitation of those bands by phosphoimager as a percentage of control (means ± SEM in seven independent experiments) following correction for loading against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The EC_{50} for ASP can be calculated at 5 nM based on these results; the EC_{50} for MSH is 10 nM (McLane et al., 1987, and our unpublished results).

To examine the time course response of melanocytes to ASP, in subsequent experiments we treated the melan-a cells with 10 nM ASP for up to 11 days (data not shown). Dramatic decreases in the levels of tyrosinase, TRP1 and TRP2 mRNAs were again noted which approached 70–98% inhibition compared with controls following treatment for 2 or more days; maximum effects were usually noted within 2 days of treatment. Again, no effects were noted on MC1-R mRNA levels during the 11 day time course of these experiments.

Interaction between ASP and MSH

As noted in the Introduction, there is evidence using MC1-R-transfected cells that ASP can act as an antagonist of
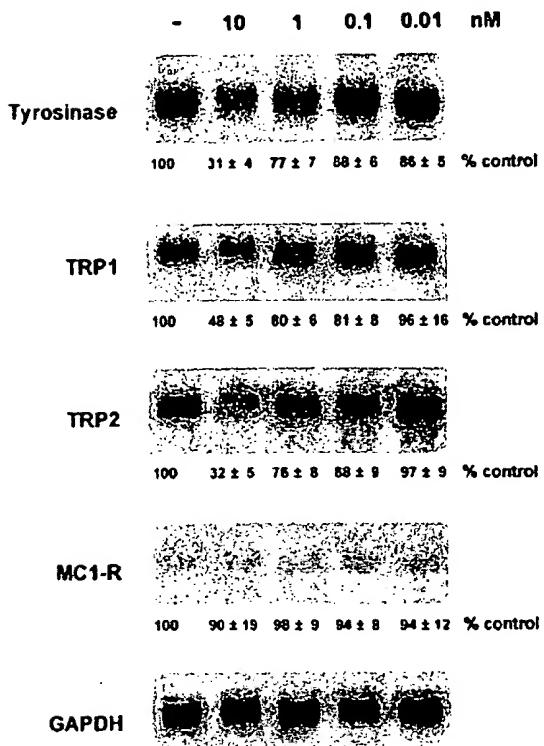

Fig. 1. Northern blot analysis of melanocytes exposed to different concentrations of ASP. Melan-a melanocytes were cultured in the absence (−) or presence of (10, 1, 0.1 or 0.01 nM) ASP for 24 h. RNA was isolated, electrophoresed, blotted to membranes and probed for expression of melanogenic genes as detailed in Materials and methods (% control corrected for loading by GAPDH is shown below each band as the mean ± SEM of seven independent experiments).

the MSH receptor. To examine directly the interaction between ASP and MSH in melanocytes, and to characterize further whether ASP might affect MSH signaling via a change in MC1-R receptor level, melan-a melanocytes were exposed to 10 nM ASP, 10 nM MSH or both for 5 days (Figure 2). This time was chosen since 4 days of treatment is the standard time to obtain the maximal response to MSH (Jiménez et al., 1988; Abdel-Malek et al., 1995) and since maximal responses to 10 nM ASP were also elicited by this time, as found in this study. We pre-treated the cells with ASP for 1 day prior to the start of MSH treatment in order to maximize the chances of seeing a competitive effect. Treatment with MSH alone produced a 2-fold increase in the level of tyrosinase mRNA, and lesser increases in TRP1 and TRP2 mRNA, but no significant change in the levels of MC1-R mRNA. On the other hand, treatment with ASP alone produced dramatic decreases in the levels of mRNAs for tyrosinase, TRP1 and TRP2, with no significant effect on the level of MC1-R mRNA. At equimolar 10 nM concentrations, addition of ASP and then MSH produced a response indistinguishable from that elicited by MSH alone, i.e. there was little or no antagonism of the MSH effect by ASP under these conditions. Measurements of the endogenous MSH concentration in the serum used for these experiments indicate that the residual MSH concen-

MSH
- MSH ASP ASP

Tyrosinase

100   198 ± 10   43 ± 13   193 ± 21   % control

TRP1

100   128 ± 6   36 ± 5   147 ± 11   % control

TRP2

100   156 ± 7   52 ± 14   179 ± 13   % control

MC1-R

100   109 ± 6   114 ± 11   111 ± 4   % control

GAPDH
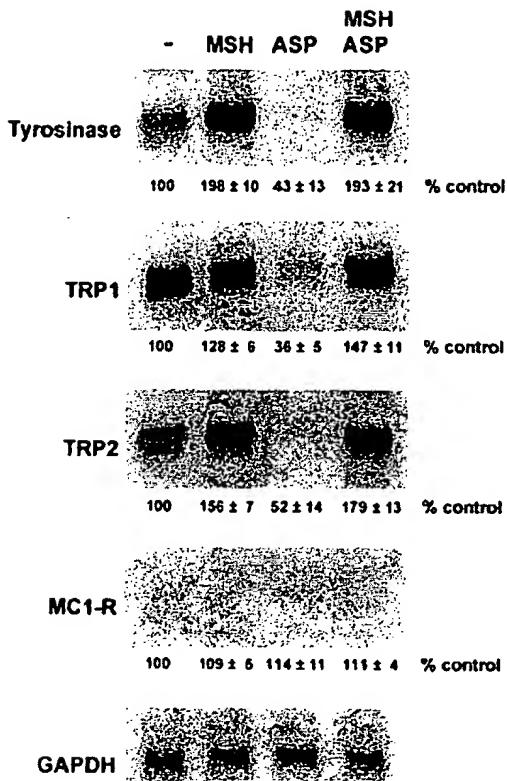
Fig. 2. Northern blot analysis of interactions between MSH and/or ASP. Melan-a melanocytes were cultured in the absence (-) or presence of 10 nM MSH, 10 nM ASP or both (MSH/ASP) for 5 days. Results are presented as detailed for Figure 1.

MSH
- MSH ASP ASP

Tyrosinase

100   332   116   275   % control

TRP1

100   189   22   141   % control

TRP2

100   176   28   120   % control

Fig. 3. Metabolic labeling and immunoprecipitation analysis of melanocytes exposed to MSH and/or ASP. Melan-a melanocytes were metabolically labeled with [^{35}S]methionine for 4 h after exposure to 10 nM MSH, 10 nM ASP or both (MSH/ASP) for 5 days. The cells were solubilized, immunoprecipitated by antibodies, separated by gel electrophoresis and visualized by autoradiography, as detailed in Materials and methods. This experiment has been repeated four times with comparable results.

tration in the medium is <2 pM, several orders of magnitude less than the EC_{50} for stimulation of tyrosinase activity or cAMP accumulation. Thus, these results confirm the ability of ASP to bring about physiologic changes in the absence of exogenously added MSH, and suggest further that the interaction of ASP and MSH is not mediated via an alteration in levels of expression of the MSH receptor itself.

Regulation of melanogenic protein expression by ASP

Steady-state levels of RNA as measured by Northern blot hybridization do not reveal alterations in the expression of gene products that might occur due to modulation of protein levels or protein function. To examine the effects of ASP at the translational level, melan-a cells were cultured in the presence or absence of MSH and/or ASP for 5 days, metabolically labeled for 6 h with [^{35}S]methionine and then subjected to immunoprecipitation analysis (Figure 3). Synthesis of tyrosinase in response to MSH treatment is significantly increased (>3-fold), while synthesis of TRP1 and TRP2 is also increased, but to a lesser extent. After exposure to ASP alone, synthesis of TRP1 and TRP2 was significantly suppressed, but we were unable to determine any inhibitory effect of ASP on tyrosinase synthesis, since, under these labeling conditions, the relatively slow synthesis of tyrosinase in the untreated

control is virtually undetectable. Exposure to MSH and ASP together produced a pattern that did not differ significantly from MSH alone, results that are consistent with those obtained by Northern blot analysis.

Melanogenic enzyme function

To examine melanogenic enzyme levels following treatment of melanocytes with MSH and/or ASP, we performed Western blotting and enzyme assays under identical conditions. Western blotting (Figure 4) revealed that there were only moderate increases in the amount of tyrosinase, and little or no change in the levels of TRP1 or TRP2, in response to MSH treatment alone. After exposure to ASP alone, there were dramatic and significant decreases in the amounts of tyrosinase, TRP1 and TRP2 proteins; note that due to its relatively long half-life, tyrosinase protein in the untreated control is readily detectable by Western blotting. Enzymatic assays revealed that the catalytic functions of tyrosinase [i.e. tyrosine hydroxylase, 3,4-dihydroxyphenylalanine (DOPA) oxidase and melanin production] were increased dramatically after exposure to MSH, but were decreased to background levels after exposure to ASP (Table I). There was little impact of MSH on the protein levels of TRP1 or TRP2 (or the enzyme activity of the latter, i.e. DOPAchrome tautomerase), whereas ASP clearly diminishes the protein levels and catalytic function of both proteins to background

MSH
- MSH ASP ASP

Tyrosinase

100 123 37 125 % control

TRP1

100 83 8 85 % control

TRP2

100 100 18 96 % control
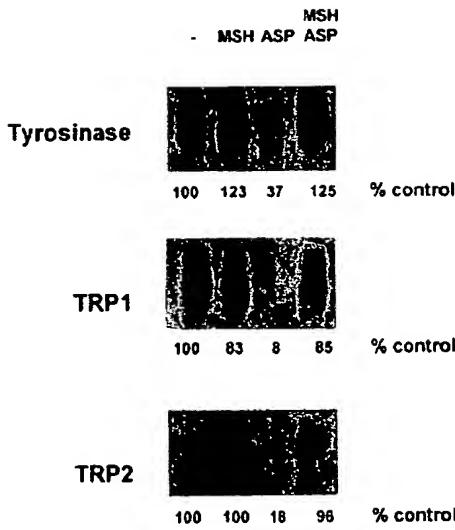
Fig. 4. Western blot analysis of expression of melanogenic proteins by melan-a cells exposed to MSH and/or ASP. Melan-a melanocytes were exposed to 10 nM MSH, 10 nM ASP or both (MSH/ASP) for 5 days; cells were then solubilized, and levels of melanogenic proteins were analyzed by Western immunoblotting, as detailed in Materials and methods.

levels. Again, simultaneous addition of both ASP and MSH produced a response indistinguishable from that elicited by MSH alone.

Structural characteristics

After incubation of melan-a cells with 10 nM ASP, the color of the cell pellets was changed from black to light brown, reflecting the decreased pigmentation evident in ASP-treated cells at the light and electron microscopic levels (Figure 5). After treatment with MSH, pigmentation of the cells was markedly increased and eumelanosomes were more numerous. However, after exposure to ASP, the number of eumelanosomes in the melan-a melanocytes was decreased significantly, and pheomelanosome-like structures were more predominant. As might be expected from the results presented above, visible pigmentation and melanosome structure of melanocytes treated with the combination of ASP and MSH were indistinguishable from those treated with MSH alone (not shown).

Chemical analysis

Chemical analysis of the types of melanins being produced in response to ASP and/or MSH reveals that while MSH treatment increases the amount of eumelanin production at least 3-fold, it elicited only a slight (~10%) but statistically insignificant decrease in pheomelanin content (Table II). Interestingly, however, treatment with ASP led to a dramatic 10-fold reduction in eumelanin content while the amount of pheomelanin produced increased slightly (~5%, but again not statistically significant).

cAMP responses

The ability of ASP to inhibit eumelanin synthesis in the absence of exogenous MSH could be mediated by an MC1-R-independent mechanism, by antagonism of residual MSH present in the culture media or by inverse

Table I. Enzyme activity of melanocytes following treatment with MSH and/or ASP

Activity	MSH only (× control)	ASP only (× control)	MSH + ASP (× control)
Tyrosine hydroxylase	20.4 ± 7.8	0.0 ± 0.1	12.6 ± 4.5
DOPA oxidase	11.4 ± 1.0	0.0 ± 0.2	11.7 ± 1.4
DOPAchrome tautomerase	1.5 ± 0.4	0.0 ± 0.2	1.4 ± 0.2
Melanin production	10.4 ± 3.0	0.1 ± 0.0	9.0 ± 2.3

Melan-a melanocytes were treated with 10 nM MSH and/or 10 nM ASP for 5 days and then were harvested and solubilized; melanogenic enzyme activities of the extracts were then measured as detailed in Materials and methods. Results for the MSH, ASP and MSH + ASP treatments are reported as n-fold of control values ± SEM ($n \geq 4$ independent experiments). Control values were: tyrosine hydroxylase, 7.4 ± 0.6; DOPA oxidase, 17.3 ± 5.1; DOPAchrome tautomerase, 148 ± 54; and melanin production, 1.2 ± 0.4 (all in pmol/μg protein/h).

agonism, i.e. a direct effect on the MC1-R itself independent of and opposite to that of MSH. In any of those mechanisms, the effects of ASP are likely to inhibit downstream effectors of cAMP such as protein kinase A, since the MC1-R is a G protein-coupled receptor which activates adenylate cyclase in vitro and in vivo (Mountjoy et al., 1992; Jackson, 1993; Suzuki et al., 1996). To determine whether ASP could bring about a decrease in cAMP levels in the absence of exogenous MSH, we measured cAMP accumulation in melanocytes exposed for 40 min to ASP alone or in combination with 10 nM MSH or 20 μg/ml cholera toxin (which increases cAMP levels by ribosylation of G_s protein). As shown in Figure 6, basal levels of cAMP accumulation in melan-a melanocytes (4.9 ± 0.1 pmol/10^6 cells) were decreased slightly by the addition of ASP at 10 nM (3.9 ± 0.3 pmol/10^6 cells) or 100 nM (3.4 ± 0.1 pmol/10^6 cells). There was a dramatic (35-fold) stimulation of cAMP following treatment of melanocytes with MSH, and this stimulation was partially inhibited by a 10-fold excess of ASP (i.e. at 100 nM ASP), and was reduced to a 13-fold increase above control. Cholera toxin elicited a 30-fold increase in cAMP accumulation, and this effect was reduced to 20- or 16-fold above control by simultaneous treatment with 10 or 100 nM ASP, respectively.

Discussion

Mammalian hair color is determined primarily by the relative proportions of eumelanin and pheomelanin produced by follicular melanocytes (Ozeki et al., 1995; Prota et al., 1995). Ratios of eumelanin:pheomelanin of >1 generally result in brown or black hair, the intensity of the color depending upon the total amount of melanins present. Eumelanin:pheomelanin ratios of <1 result in the production of yellow or red hair. By those criteria, the melanins being produced by melan-a melanocytes under basal conditions, or following treatment with MSH, would be black or brown (i.e. eumelanic) while those produced following treatment with ASP would be yellow or red (i.e. pheomelanic). This result is quite reasonable since melan-a melanocytes were generated from non-agouti black mice and, in the absence of ASP, would be expected to produce eumelanin. Conversely, treatment of melan-a cells with ASP would be expected to induce pheomelano-
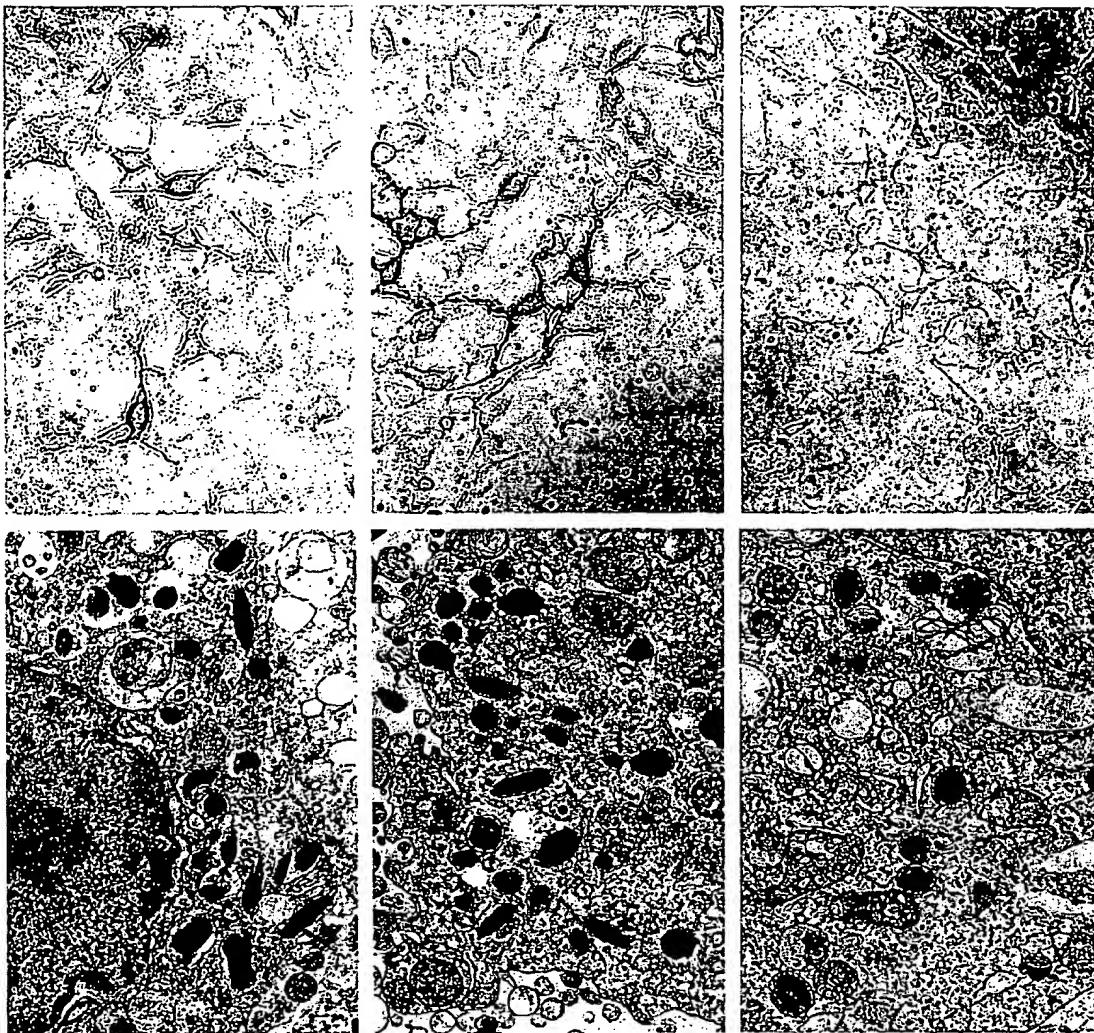
Fig. 5. Light and electron microscopy of melanocytes treated with MSH and/or ASP. Light (top row, all at initial magnification of 200×) and electron (bottom row, all at initial magnification of 80 000×) microscopy of melan-a melanocytes that were untreated (left), treated with 10 nM MSH (center) or with 10 nM ASP (right) for 5 days. Many melanosomes are found in the untreated control and the MSH-treated cells; at higher magnification, it can be seen that most have the typical elliptical shape with striated filaments that are characteristic of eumelanosomes. Following treatment with ASP for 5 days, the numbers of melanosomes are significantly decreased, and many have pheomelanosome-like ovoid shapes with a more particulate internal matrix.

genesis *in vivo*, and this occurs in tissue culture as well, suggesting that this *in vitro* system is an appropriate one mimicking physiological conditions.

Much is known about the biochemistry and cell biology of eumelanogenesis, but a similar level of understanding has not been achieved for pheomelanogenesis due, in part, to the lack of an appropriate cell culture system. *In vivo*, MSH promotes the production of eumelanin, while expression of the *agouti* gene promotes the production of pheomelanin. In this study, we now show that purified recombinant ASP added to melan-a melanocytes in culture decreases the expression of several eumelanogenic genes, reduces the production of eumelanin and eumelanosomes and increases the production of pheomelanin and pheomelanosome-like structures. These results establish an important tool with which to study pheomelanogenesis and, in addition, suggest that the biochemical action of

MSH is not completely reciprocal to that of ASP. MSH stimulates the expression and function of tyrosinase but has little or no effect on TRP1 or TRP2; ASP on the other hand down-regulates all three of these melanogenic enzymes.

The mechanism by which ASP acts remains controversial (Conklin and Bourne, 1993; Jackson, 1993; Zemel *et al.*, 1995). While ASP clearly acts as a competitive antagonist of the MC1-R in heterologous cells, this mechanism alone cannot easily explain why ubiquitous overexpression of the *agouti* gene *in vivo* in *lethal yellow* mice affects pigmentation and regulation of body weight differently from *recessive yellow* mice which have a loss-of-function mutation of the MC1-R. Much of the controversy stems from the lack of a suitable *in vitro* assay system that accurately reflects the biological activity of the protein *in vivo*. Our results not only provide such

Table II. Chemical analysis of melanins following treatment with
MSH and/or ASP

	MSH only (× control)	ASP only (× control)	MSH + ASP (× control)
Pheomelanin content	0.91 ± 0.13	1.05 ± 0.02	0.84 ± 0.10
Eumelanin content	3.11	0.10	2.55
Eumelanin/pheomelanin	3.42	0.09	3.04

Melanins produced in the samples as described for Table I were
subjected to chemical analysis as detailed in Materials and methods;
pheomelanin content is estimated by analysis of aminohydroxyphenyl-
alanine (AHP) derivative in the degraded sample, whereas eumelanin
content is estimated by quantitation of the pyrrole-2,3,5-tricarboxylic
acid (PTCA) derivative. In each experiment, AHP analyses were
performed routinely in duplicate, whereas PTCA analyses could only
be performed once due to the sample size required. Results for the
MSH, ASP and MSH + ASP treatments are reported as n-fold of
control values (averages of two independent measurements). Control
values were: pheomelanin content, 169 ng/mg protein; eumelanin
content, 179 ng/mg protein.

Fig. 6. Effects of ASP, MSH and cholera toxin on cAMP
accumulation. Melan-a melanocytes were cultured to subconfluence,
then incubated for 40 min in the presence of MSH (at 10 nM), cholera
toxin (CT, at 20 µg/ml) and/or ASP (at 10 or 100 nM), then assayed
for cAMP concentration as detailed in Materials and methods; results
are reported as pmol/10^6 cells ± SEM (n = 6).

an assay system, but also shed insight into the underlying
mechanism, since the ability of purified ASP to induce
pheomelanogenesis in eumelanic melanocytes demon-
strates that ASP alone is sufficient to elicit such changes
and that cytokines, growth factors, endothelins and other
constituents of the epidermis or hair bulbs are not required.
This study now confirms the antagonism of ASP on MSH-
induced stimulation of cAMP in melanocytes, but also
shows that ASP has physiologic effect(s) in the absence
of exogenous MSH.

Two of the genes we have examined, TRP1 and TRP2,
encode enzymes that catalyze specific distal steps in the
eumelanogenic pathway, and we have shown previously
that expression of these genes ceases during pheomelano-
genesis *in vivo*. The enzymatic activity of the third member
of the TRP family, tyrosinase, is required for both types
of pigment synthesis; tyrosinase is also down-regulated
during pheomelanogenesis *in vivo*, though not to the same
extent as TRP1 and TRP2. The changes in gene expression
we have described here for melan-a cells treated with ASP
are similar to those that occur during pheomelanogenesis
in vivo, although *in vitro* ASP has a comparable down-
regulatory effect on tyrosinase, TRP1 and TRP2. This
discrepancy may be caused by intrinsic differences
between melan-a cells, which were derived from neonatal
epidermis, and hair follicle melanocytes. Epidermal and
hair follicle melanocytes arise from the same pool of
precursor melanoblasts, but could later acquire changes
in cell surface receptors or intracellular signaling that affect
their pheomelanogenic potential. In addition, extrinsic
differences could help to explain why pheomelanin is
produced within and not between hair follicles, and
treatment of melan-a cells with paracrine factors unique
to the follicular microenvironment may identify molecules
that function in addition to and/or downstream of ASP
that promote pheomelanogenesis.

The results of chemical analyses of melanins produced
in the presence or absence of MSH and/or ASP *in vitro*
clearly demonstrate the effect of MSH in stimulating
eumelanin synthesis with a negligible effect on pheomel-
anin production. MSH thus elicits a marked increase in the
percentage of eumelanin produced and thus dramatically

decreases the percentage contribution of pheomelanin to
the total melanin. ASP, on the other hand, caused a
significant decrease in eumelanin production while at the
same time slightly stimulating pheomelanin synthesis,
resulting in a dramatic increase in the percentage contribu-
tion of pheomelanin (91%) to the total melanin content.
The lack of correlation of pheomelanin production with
tyrosinase mRNA synthesis or enzyme function argues
strongly that an as yet undiscovered enzyme or regulatory
point is at least partly responsible for pheomelanin content.
Why eumelanogenic melanocytes, when introduced into
culture, produce significant levels of pheomelanin in the
absence of exogenously added ASP, is not known at this
time, although it has been noted previously (Sato *et al.*,
1985a; Hunt *et al.*, 1995). The latter report showed that
human melanocytes introduced into culture produced sig-
nificant levels of pheomelanin that did not correlate with
the racial origin of those melanocytes. It is possible that
nutrient concentrations in the media that are not present
in the epidermal microenvironment play a role in this
determination, but further study will be necessary to
resolve this point.

Several hypotheses have been put forward to explain
the mechanism of ASP action, including competitive
antagonism for MSH binding, binding to an as yet unidenti-
fied 'agouti receptor' or modulation of calcium flux.
Distinguishing between these alternatives is difficult since
virtually all of the effects of ASP, including those reported
here, can be reversed by the addition of exogenous MSH.
Our results demonstrate that ASP antagonizes MC1-R
signaling, but effects of ASP treatment can be observed
in the absence of exogenous MSH. Our findings are
unlikely to be explained by the presence of residual
MSH in the culture medium since the concentration of
endogenous MSH in the medium is 1000-fold below the
EC_{50} for MSH, but could be accounted for by inverse
agonism, particularly if the MC1-R has a significant degree
of constitutive activity. The hypothesis that the action of
ASP is independent of MSH but not of the MC1-R could
be addressed by studies of normal melanocytes cultured
from non-mutant animals and those that carry the *recessive
yellow* (*Mc1r^e*) mutation. The stage is now set for critical
studies to characterize the regulation of gene expression

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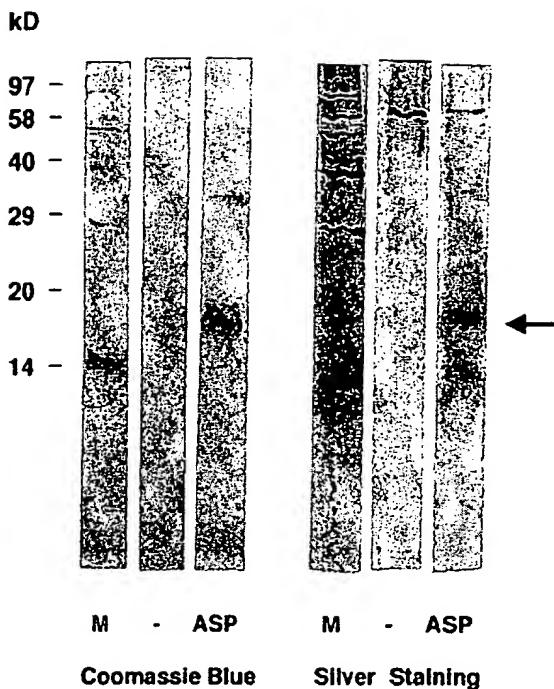
Fig. 7. Purity of recombinant ASP used in these studies. ASP was expressed in a baculovirus system, purified and separated by SDS electrophoresis on 15% acrylamide gels as detailed in Materials and methods. The position of molecular weight markers (M) is shown, as is a buffer control (–); 1 µg of ASP was used on the Coomassie Blue-stained gel (left) while 0.1 µg of ASP was used in the silver-stained gel (right).

and cellular signaling mechanisms triggered by MSH and ASP that modulate the pheomelanogenic switch in melanocytes.

Materials and methods

Cells and cell culture conditions

The melan-a melanocyte line, derived from C57B1 non-agouti black mice, was a kind gift from Dr Dorothy Bennett, London, and was cultured in Dulbecco's minimal essential medium with additives as described by Bennett et al. (1987). Cells were usually seeded at 1.5×10^6 cells per 15 cm diameter dish. For 24 h experiments, we added ASP and/or MSH immediately, while for the 5 day experiments, we added ASP immediately, and MSH was added starting on the next day. The concentrations of ASP and MSH used ranged from 0.01 to 10 nM, as detailed in the figure and table legends. The cells were cultured routinely at 37°C in a humidified incubator with 5% CO_2, and all media were changed daily. Cells were harvested by brief treatment with trypsin/EDTA, and used for subculture, or were processed for RNA, protein or enzyme analysis, as detailed below.

Agouti signal protein

Recombinant mouse ASP was generated and purified using a baculovirus expression system as described in Ollmann et al. (in preparation). The ASP used for the experiments is ≥90% pure, as estimated by analysis of gels stained with Coomassie Blue or silver stain (Figure 7), and inhibits activation of the MC1-R with a K_i of 2.2×10^{-10} M. At 37°C, ASP retains activity for >48 h in water or tissue culture media. The experiment described in Figure 2 has also been repeated with an ASP preparation ≥99% pure with virtually identical results.

Electron microscopy

Cells were harvested, centrifuged for 5 min at 14 000 g at 4°C, and fixed for 2 h at 23°C in 2% glutaraldehyde–2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. The samples were stored in phosphate-buffered saline (PBS) containing 2% sucrose at 4°C, then processed with graded alcohols and embedded in epoxy resin in the usual manner. Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed with a Zeiss EM10 electron microscope, as previously detailed (Prota et al., 1995).

RNA isolation and Northern blotting

Total RNA was extracted from cells using an RNeasy total RNA isolation kit (QIAGEN, Catsworth, CA), following the manufacturer's instructions. Twenty µg of total RNA were denatured, electrophoresed through 1.0% agarose gels and transferred overnight at 23°C to SureBlot nylon hybridization membranes (Oncor, Gaithersburg, MD) in the standard manner. Filters were pre-hybridized for 3 h at 45°C with Hybrisol I solution (Oncor), and then hybridized with ^{32}P-labeled probes. A 2.0 kb EcoRI fragment of TYRS-J, a 1.7 kb HindIII fragment of pMT4, a 1.75 kb EcoRI fragment of TRP2a and a 2.1 kb BamHI–SalI fragment of A26 were used to detect tyrosinase, TRP1, TRP2 and MC1-R mRNAs, respectively. TYRS-J was obtained from Drs Hiroaki Yamamoto and Takuji Takeuchi, Sendai, Japan; pMT4 was obtained from Dr Shigeki Shibahara, Sendai, Japan; TRP2a was obtained from Dr Ian Jackson, Edinburgh, UK; A26 was obtained from Dr Roger Cone, Oregon. A commercially available cDNA probe specific for GAPDH was used to standardize RNA loading on the blots. The cDNA probes were labeled using random primer extension and heated to 100°C for 10 min, then cooled on ice for 10 min prior to adding to the hybridization solution. Hybridization was performed with the radiolabeled probes in Hybrisol I (3×10^7 c.p.m./10 ml) overnight at 45°C with gentle shaking. Following incubation, the blots were washed for 10 min at 23°C with 2× SSC/10% SDS, for 10 min with 0.2× SSC/0.5% SDS and finally for 10 min with 0.1× SSC/0.1% SDS. Blots were exposed in phosphoroimager cassettes at 23°C for 1 h and the densities of the bands were scanned using ImageQuant software. The percentage control for each probe was corrected for initial loading using comparison with the GAPDH standard. Residual probes were then removed by repeated washings for 15 min at 100°C in 0.1× SSC/0.1% SDS in 10 mM Tris, pH 7.0 until no remaining probe could be detected.

Metabolic labeling and immunoprecipitation

These techniques were performed as previously reported (Tsukamoto et al., 1992; Aroca et al., 1993). Briefly, subconfluent cells growing in 10 cm diameter dishes were pre-incubated for 1 h at 37°C in methionine-free medium, and then radiolabeled for 6 h with 0.4 mCi/flask of [^{35}S]methionine. The cells were then harvested and solubilized for 1 h at 4°C with NP-40/SDS buffer (1% NP-40, 0.01% SDS, 0.1 M Tris–HCl, pH 7.2, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin). The cell lysates were then centrifuged for 15 min at 14 000 g at 4°C, and the supernatants were pre-treated overnight at 4°C with normal rabbit serum and GammaBind G Sepharose (Pharmacia/LKB, Piscataway, NJ) to reduce background. Then 5×10^6 c.p.m. of each pre-absorbed supernatant were incubated with 10 µl of rabbit antibodies generated against peptides corresponding to the unique carboxyl sequences of the three melanogenic proteins studied; they are termed αPEP1, αPEP7 and αPEP8, which recognize TRP1, tyrosinase and TRP2, respectively (Tsukamoto et al., 1992). Following incubation at 37°C for 1 h, 50 µl of GammaBind G Sepharose was mixed in each tube for 20 min at 23°C, and the immune complexes were washed four times with NP-40/SDS buffer at 23°C and then denatured in SDS sample buffer by heating to 100°C for 3 min. Specifically bound proteins were then analyzed by SDS–PAGE and visualized by autoradiography.

Western immunoblotting analysis

Cells in tissue culture were harvested and solubilized for 1 h at 4°C with NP-40/SDS buffer, then centrifuged at 14 000 g for 15 min at 4°C, and the supernatants were recovered. Proteins from the NP-40/SDS-solubilized cells were separated on 7.5% SDS gels, and then transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). Following blocking overnight at 23°C in 3% bovine serum albumin in TBS/Tween (0.1% Tween-20 in Tris-buffered saline), the blots were incubated with primary antibodies (at 1:1000 dilution in TBS/Tween). Following four washes in TBS/Tween to reduce non-specific binding, subsequent visualization of specific antibodies bound was carried out with Enhanced ChemiLuminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Melanogenic enzyme assays

Melanogenic assays were carried out routinely on NP-40/SDS-soluble extracts (obtained as above) at pH 6.8, 37°C for 60 min. To determine tyrosinase activity, the tyrosine hydroxylase assay was used to measure tritiated water produced during the hydroxylation of L-[3,5-^{3}H]tyrosine to DOPA (Hearing, 1987). For DOPA oxidase activity, the production of acid-insoluble melanin product from [3-^{14}C]DOPA was measured (Aroca et al., 1993). To determine DOPAchrome tautomerase activity, the disappearance of DOPAchrome substrate and the production of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than 5,6-dihydroxy-indole (DHI) was measured by HPLC (Tsukamoto et al., 1992). To determine melanin production, the [U-^{14}C]tyrosine assay (Hearing, 1987) was used. All radioactive precursors were obtained from DuPont-New England Nuclear. DOPAchrome was prepared using the silver oxide method, and DHI and DHICA used as standards were obtained from Pierce Chemical Co. (Rockford, IL) and from Professor Shosuke Ito, Nagoya, Japan. The pmoles of product of the assays were calculated from radioactivity measured or by comparison with standard curves.

cAMP assays

These assays were performed as previously detailed (Suzuki et al., 1996). Briefly, cells were plated into 24-well plates at 3×10^5 cells per well and allowed to grow for 48 h with a single change of medium. The media were then removed from each well, and the cells were incubated for 40 min in the presence of MSH, ASP and/or cholera toxin, following which the reactions were stopped with 1 M HCl. Each sample was then acetylated by the addition of triethylamine and acetic anhydride, and the amount of cAMP was determined by radioimmunoassay as previously detailed (Liggett et al., 1989).

Chemical analysis

Chemical degradation and analysis of eumelanin and pheomelanin contents were performed and quantitated as previously reported (Ito and Fujita, 1985).

Acknowledgements

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SURFACE ACTIVE AGENTS

THEIR CHEMISTRY AND TECHNOLOGY

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PREFACE

This book is an attempt to summarize the achievements of the last three decades in developing a host of new surface active agents. The different types of products are reviewed with particular attention to their laboratory synthesis, commercial production, and characteristic properties. Special attention has been directed to the numerous practical applications of surface active agents and to their utilitarian effects—foaming, wetting, detergency, emulsification, spreading, etc. In order to provide background for a better understanding of the diverse practical applications and related effects, the fundamentals of the chemistry and physics of surface phenomena have been summarized separately.

It was our aim to present a well-integrated picture of the present state of development of surface active agents. It is hoped that this book may prove interesting and helpful to practicing chemists in general, to advanced students in chemistry and chemical technology, and particularly to specialists in the production, investigation, and application of surface active agents.

Because the mass of factual data pertinent to our subject is too large to be encompassed within a single volume, numerous references have been included both to the original literature and to reviews of various special aspects of the broad field.

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September, 1948

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Inhibition of Melanosome Transfer Results in Skin Lightening[1]

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The chemical basis of melanogenesis is well documented, but the mechanism of melanosome transfer and the regulation of pigmentation by keratinocyte–melanocyte interactions are not well understood. Therefore we examined the effects of serine protease inhibitors on skin pigmentation and found that the protease-activated receptor 2, expressed on keratinocytes, may regulate pigmentation via keratinocyte–melanocyte interactions. Here we show that modulation of protease-activated receptor 2 activation affects melanosome transfer into keratinocytes, resulting in changes in pigment production and deposition. SLIGRL, the protease-activated receptor 2 activating peptide, enhanced melanosome ingestion by keratinocytes, thus increasing pigment deposition. RWJ-50353, a serine protease inhibitor, led to reduced pigment deposition in melanocytes and depigmentation. Electron microscopy studies illustrated an accumulation of immature melanosomes inside melanocytes and abnormal dendrite dynamics in RWJ-50353-treated epidermal equivalents. RWJ-50353 induced a visible and dose-dependent skin lightening effect in the dark-skinned Yucatan swine. Examinations by electron microscopy indicated that the *in vivo* transfer of melanosomes from melanocytes to keratinocytes was affected. Our data suggest that modulation of keratinocyte–melanocyte interactions via the protease-activated receptor 2 pathway affects melanosome transfer. The use of RWJ-50353 to modulate protease-activated receptor 2 activation could lead to a new class of depigmenting agents. *Key words: PAR-2/RWJ-50353. J Invest Dermatol 115:162–167, 2000*

M elanin synthesis within melanosomes and their distribution to keratinocytes within the epidermal melanin unit determine skin pigmentation. The essential role of keratinocytes in the regulation of melanocyte growth and differentiation has been demonstrated (Donatien *et al*, 1993) but the regulation of keratinocyte–melanocyte interactions and the mechanism of melanosome transfer into keratinocytes are not yet fully understood.

Concerns of changes in skin color are frequently raised for medical or cosmetic reasons. Pigmentary disorders can be inherited (e.g., vitiligo, Waardenburg syndrome), acquired (e.g., postinflammatory pityriasis alba, idiophatic guttate hypomelanosis, melasma), medication related (e.g., minocycline, bleomycin, busulfan, zidovudine), and transmitted through infection (e.g., tinea versicolor). Changes in skin color are also desired for cosmetic reasons. Hyperpigmentation disorders are often treated with hydroquinones, retinoids, and tyrosinase inhibitors, but results of such treatments are sometimes disappointing (Hacker, 1996).

The protease-activated receptor 2 (PAR-2) (Nystedt *et al*, 1994, 1995a) is a seven transmembrane G-protein-coupled receptor that is activated by a serine protease cleavage. The newly created N-

terminus then activates the receptor as a tethered ligand. PAR-2 is also activated by SLIGRL, the peptide that corresponds to its new N-termini, independent of receptor cleavage (Nystedt *et al*, 1995b; Bohm *et al*, 1996). PAR-2 is expressed in skin (Santulli *et al*, 1995), but its biology is not yet completely understood. A role for PAR-2 activation in the inhibition of keratinocyte growth and differentiation has been suggested (Derian *et al*, 1997). We have recently demonstrated that the PAR-2 pathway is involved in the regulation of pigmentation (Seiberg *et al*, 2000).

Here we show that serine protease inhibitors that interfere with PAR-2 activation induce depigmentation by affecting melanosome transfer and distribution. Such agents may serve as an alternative treatment for depigmentation.

MATERIALS AND METHODS

Cells and cultures Epidermal equivalents containing melanocytes (equivalents) (MelanoDerm) were from MatTek (Ashland, MA) and were maintained according to the manufacturer's instructions. Murine Melan-A cells (a kind gift from Dr. D. Bennett) were maintained according to Bennet *et al* (1987). Human HaCaT keratinocytes (a kind gift from Dr. N. Fusenig) were maintained according to Boukamp *et al* (1988). Cell growth and viability were assayed using an MTS proliferation assay kit (Promega, Madison, WI) and alamarBlue (Accumed International, Chicago, IL), respectively, following the manufacturers' instructions. No SLIGRL and RWJ-50353 treatments resulted in no change in cell viability or proliferation (not shown). RWJ-50353 (Costanzo *et al*, 1996) and SLIGRL were dissolved in phosphate-buffered saline (PBS) for *in vitro* studies. All *in vitro* experiments were performed in triplicate and were repeated at least three times.

## Control   RWJ-50353   SLIGRL

Figure 1. The effect of RWJ-50353 and SLIGRL on pigmentation in individual melanocytes. Equivalents were treated with SLIGRL (10 μM) and with RWJ-50353 (0.1 μM) for 3 d, followed by F&M staining of histologic sections. Melanocytes were from a Hispanic donor. Images of individual melanocytes are shown. *Left panels*, untreated control; *middle panels*, RWJ-50353; *right panels*, SLIGRL. *Scale bar*, 10 μm.
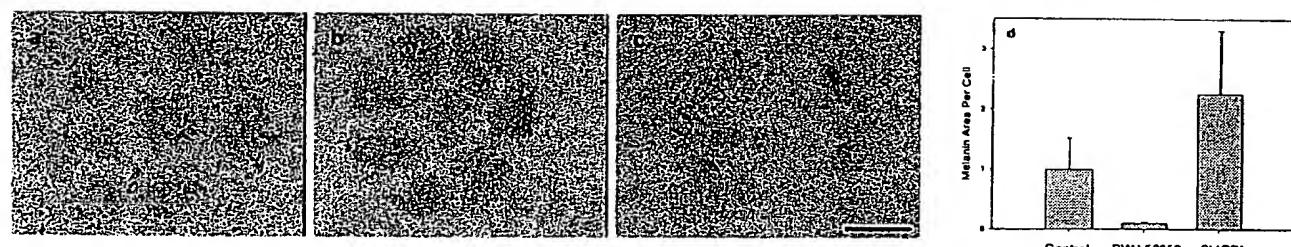

Figure 2. The PAR-2 pathway affects melanosome ingestion by keratinocytes. Melanosomes were isolated from Melan-A cells according to Orlow *et al* (1994). Images of HaCaT keratinocytes untreated (*a*) or treated with SLIGRL (10 μM, *b*) or RWJ-50353 (10 nM, *c*) for 2 d, followed by a 2 h incubation with the isolated melanosomes, extensive wash, and F&M staining. *Scale bar*, 10 μm. (*d*) Melanin area per cell, quantified by image analysis.

Melanosome isolation and transfer Melanosome isolation was performed according to Orlow *et al* (1994). Briefly, Melan-A cells were harvested in PBS supplemented with 10% (wt/vol) glucose (Sigma) and protease inhibitors (Complete, Boehringer Mannheim, Indianapolis, IN). Cell suspensions were homogenized on ice until cells were disrupted, and centrifuged at 500*g*, 10 min (4°C) to remove nuclei. Supernatants were supplemented with 90% (vol/vol) Percoll (Sigma) suspended in 0.25 M sucrose buffer (10 mM HEPES, 1 mM ethylenediamine tetraacetic acid, pH 7.2) to yield a final 28% Percoll concentration and were centrifuged at 10,000*g*, 45 min (4°C). Melanosome band was removed with a 25 gauge needle and syringe and stored at 4°C until use. Pretreated HaCaT keratinocytes were incubated with the isolated melanosomes for 2 h, followed by 10 PBS washes and Fontana-Mason (F&M) staining.

Histology, image analysis, and electron microscopy Sections from swine biopsies and equivalents were stained with hematoxylin and eosin (not shown) or F&M, and monolayer cultures were stained with F&M (Sheehan and Hrapchak, 1980). F&M detects silver nitrate reducing molecules, which in skin identifies primarily melanin. At least three sections per equivalent or biopsy, three equivalents per experiment, were processed. At least 100 cells were used for each melanosome transfer group. Each experiment was repeated three times. Data are presented as the average of all experiments, with standard deviation (SigmaPlot 5.0, SPSS Science,

Chicago, IL). F&M- or dihydroxyphenylalanine-stained sections, intact equivalents, or monolayers were used for image analysis. Empire Images database 1.1 was used on a Gateway 2000 P5-100 computer (Media Cybernetics, Silver Springs, MD). Image Pro plus version 4.0 was used for capturing images and image analysis. Parameters measured were the surface area of silver deposits and the tissue area or cell number, and the ratio of silver deposits per area or per cell was calculated. A value of one was assigned to untreated controls, and values of treatment groups were normalized to their relevant controls. Statistical analysis was done using SigmaStat 2.0 (SPSS Science) software. In all experiments there was no difference between PBS-treated equivalents and untreated controls (not shown). Electron microscopy was performed using standard protocols, as described by Piekos (1989).

Animals Yucatan swine were from Charles River (Maine). Swine were housed in appropriately sized cages in an environmentally controlled room with a 12 h light, 12 h dark photoperiod and were supplied with food and water *ad libitum*. Animal care was based on the *Guide for the Care and Use of Laboratory Animals*, NIH Publication 85–23. Test compound or vehicle (ethanol:propylene glycol mix, 70:30 wt/wt) was applied topically, twice a day, 5 d per wk, for 8–9 wk. Treatments of individual swine were always arranged in a head to tail order on one side, and in a tail to head order on the other side of the animal. Color measurements of treated sites and nearby
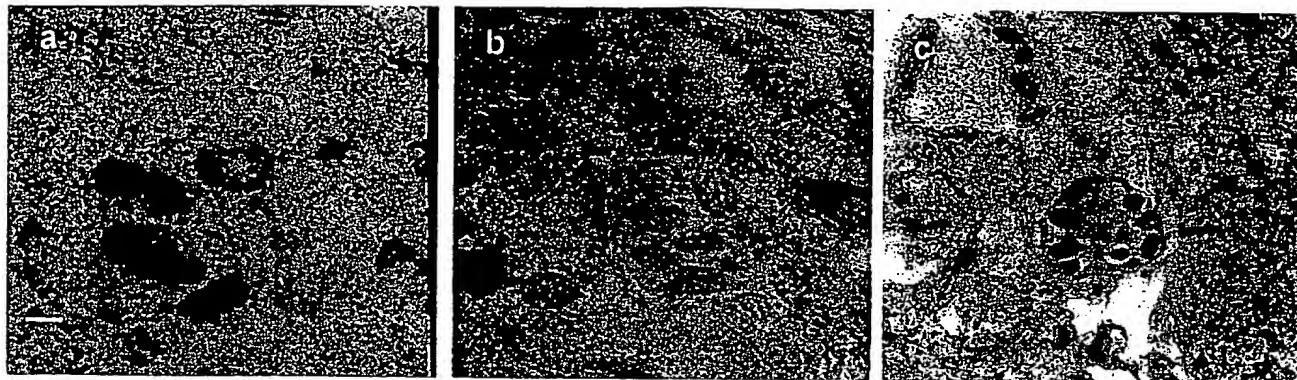
Figure 3. Electron microscopy analysis of the RWJ-50353 effect on equivalents. Representative melanosomes as identified in (a) control and (b) RWJ-50353 (100 nM) treated equivalents. (c) A melanocyte dendrite, containing melanosomes, inside an RWJ-50353-treated keratinocyte. Such structures could not be easily identified in untreated controls. Scale bar: (a, b) 0.1 μm; (c) 0.5 μm.
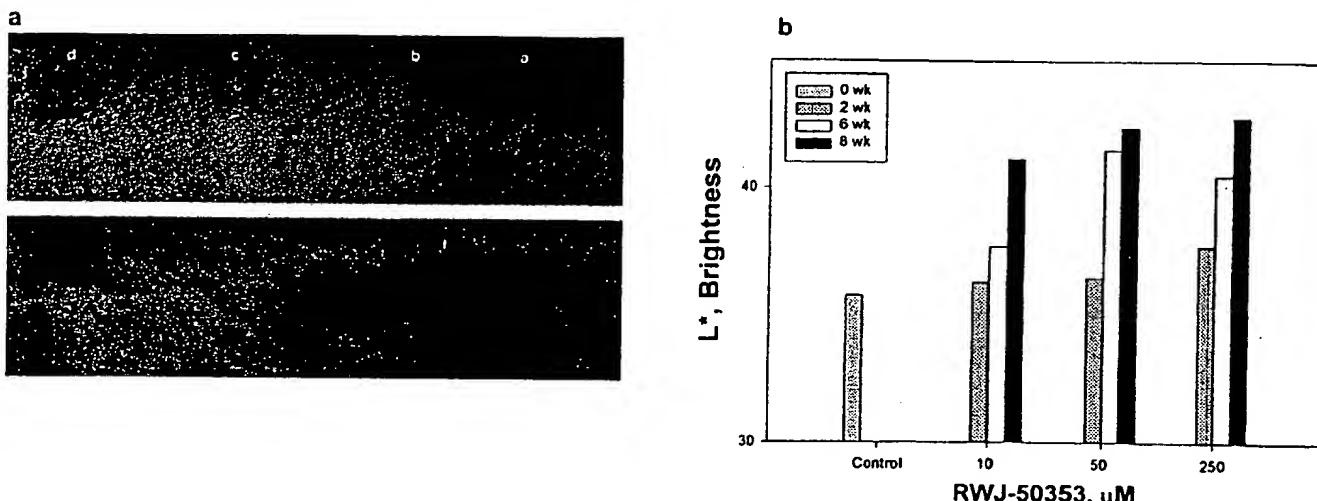

Figure 4. RWJ-50353-induced depigmentation *in vivo*. Yucatan swine were treated with vehicle (a), and 10 μM (b), 50 μM (c), and 250 μM (d) of RWJ-50353 for 8 wk. (a) Picture of the swine (both sides) after 8 wk of treatment. (b) Chromameter measurements of skin color (L* scale, 0 = black, 100 = white) during the treatment phase. Both a dose response and a time response are observed.

untreated regions were taken before the start of treatments and every 2 wk, using a Minolta Chromameter model CR300 (Osaka, Japan). Biopsies were taken using standard techniques. Swine experiments were repeated with at least three individual swine. The reversal of depigmentation effect was repeated twice.

RESULTS

PAR-2 affects pigmentation via modulation of melanosome uptake Our previous studies suggest that the PAR-2 pathway affects pigmentation via keratinocyte–melanocyte interactions (Seiberg et al, 2000). As shown in Fig 1, treatment of multilayered equivalents with the PAR-2 peptide agonist SLIGRL induces pigmentation in individual melanocytes. Treatment with RWJ-50353 (Costanzo et al, 1996), a serine protease inhibitor that affects PAR-2 activation, results in decreased pigmentation. As PAR-2 is expressed in keratinocytes but not in melanocytes and as keratinocyte-melanocyte contact is required for the PAR-2 effect on pigmentation (Seiberg et al, 2000), we tested the possible role of the PAR-2 pathway in melanosome uptake. Melanosomes isolated from Melan-A cells (according to Orlow et al, 1994) were incubated with HaCaT keratinocytes that were pretreated for 2 d with SLIGRL or RWJ-50353. As shown in Fig 2(a)–(c), an increase in uptake of melanosomes by the keratinocytes was observed following SLIGRL treatment.

Ingestion of melanosomes was inhibited following RWJ-50353 treatment. Image analysis of the melanin area within the keratinocytes (Fig 2d) showed a 2.2-fold increase in melanosome uptake following PAR-2 activation, and an about 80% decrease following RWJ-50353 treatment (p = 0.006 and 0.002, respectively, t test). These results suggest that the keratinocyte PAR-2 is involved in melanosome uptake.

Ultrastructural analysis of the RWJ-50353 effect Equivalents treated with RWJ-50353 were analyzed for melanosome formation and distribution using electron microscopy. As shown in Fig 3, melanosomes in melanocytes of the treated samples (Fig 3b) were less mature and increased in number, relative to untreated controls (Fig 3a). Dendrites containing mature melanosomes were identified within treated keratinocytes (Fig 3c) in higher numbers (nine in 27 fields) than in untreated controls (one in 25 fields). These data suggest abnormal melanosome formation and slow or impaired melanosome transfer into keratinocytes in the treated samples.

A dose-dependent *in vivo* whitening effect of RWJ-50353 Pigmented Yucatan swine were topically treated with RWJ-50353 twice daily for 8 or 9 wk. Skin color was measured by chromameter before the start and throughout the treatment period. A visible whitening effect was observed starting at the fourth week (highest dose). By 8 wk all RWJ-50353-treated sites exhibited

Figure 5. Histologic analysis of swine skin samples treated with RWJ-50353. Swine were treated as described in Fig 4 and biopsies were taken at the eighth week of treatment. F&M staining of skin sections revealed a dose-dependent reduction in melanin deposition at treated sites: (a) vehicle; (b)–(d) 10 μM, 50 μM, and 250 μM of RWJ-50353. Scale bar. 20 μm. (e) Relative pigmentation, calculated as melanin area per epidermis area and normalized to untreated controls, obtained by image analysis of F&M-stained skin sections.

whitening, with a saturation effect for the two highest doses (Fig 4a). Chromameter measurements throughout this study (Fig 4b) showed that the saturation effect is time and concentration dependent. No signs of irritation were observed during the course of treatment.

Histologic analysis of skin biopsies taken at the eighth week of treatment further confirmed the depigmenting effect of RWJ-50353 (Fig 5a–d). Reduced pigment deposition is observed in RWJ-50353-treated skin throughout the basal layer, as well as suprabasally. No other changes were observed in the treated sections, skin architecture was intact, and no inflammatory infiltrate was detected. Image analysis of F&M-stained sections (Fig 5e) demonstrated the dose-response lightening effect of RWJ-50353, with statistically significant changes at 50 and 250 μM (p = 0.003, p < 0.001, respectively, t test).

The lightening effect of RWJ-50353 was reversible. Yucatan swine treated for 8 wk were followed visually with no treatment for a further 4 wk. Darkening of the depigmented sites was visible by the fourth week. Histologic analysis (Fig 6a–e) showed a gradual increase in pigment production and distribution even before the visual observation of re-pigmentation. Image analysis of F&M-stained sections (Fig 6f) quantified this gradual re-pigmentation. Statistical analysis (t test) showed significant lightening (p = 0.003) following 8 wk of RWJ-50353 treatment (relative to control), and a slow increase in re-pigmentation weekly, which became statistically significant (relative to the 8 wk treatment time point) only after 4 wk (p = 0.003).

Ultrastructural analysis of RWJ-50353-treated skin Skin samples from Yucatan swine treated with RWJ-50353 for 8 wk were analyzed by electron microscopy. Melanosomes within keratinocytes of treated sites were 30%–40% smaller and less pigmented compared with controls (Fig 7a–c). Only single melanosomes, and no melanosome complexes, were observed in either the control or the treated sites. Moreover, the distribution of melanosomes within the treated skin was abnormal. Melanosomes were detected mainly at the epidermal–dermal border, compared to a more random distribution in the untreated controls (Fig 7d, e).

DISCUSSION

Various pigmentary disorders and cosmetic applications require the use of depigmenting agents. Currently available topical agents used

for the reduction of pigmentation include tyrosinase inhibitors and melanocyte-cytotoxic agents (reviewed in Jimbow and Jimbow, 1998). Although advances have been made, there is currently a need for safer, more effective, and less irritating depigmenting therapies. Basic understanding of the regulation of pigment production and distribution could aid in the identification of alternative depigmenting agents.

The process of melanogenesis is well studied. Melanin is produced within melanosomes, which later migrate into the melanocyte's dendrite tips using myosin V filaments (Wei et al, 1997) and a dynein "motor" (Ogawa et al, 1987). The regulation of pigment production by keratinocyte–melanocyte interactions and the subsequent transfer and distribution of pigment into keratinocytes, however, are not well understood. Several mechanisms for melanosome transfer from the dendrite tips into the keratinocytes have been suggested, including phagocytosis, release of melanosomes into intercellular spaces followed by endocytosis, direct inoculation ("injection"), and keratinocyte-melanocyte membrane fusion. No molecular mechanism has been identified for melanosome transfer (reviewed in Yamamoto and Bhawan, 1994; Jimbow and Sugiyama, 1998).

As PAR-2 modulation affects pigmentation only when a keratinocyte-melanocyte contact is established (Seiberg et al, 2000), we looked at the effect of the PAR-2 pathway on melanosome uptake by keratinocytes. Here we show the first molecular mechanism involved in melanosome uptake, as PAR-2 affecting agents regulate the ingestion of melanosomes by keratinocytes in culture. Preliminary data not presented here show that PAR-2 affects keratinocyte ingestion of microspheres and Escherichia coli particles, suggesting a role for PAR-2 in keratinocyte phagocytosis. The keratinocyte receptor PAR-2 could therefore be a part of the regulatory mechanism of skin pigmentation. Synthetic compounds that affect the PAR-2 pathway are shown to modulate melanosome ingestion. RWJ-50353, a serine protease inhibitor that reduced melanosome uptake in culture, is shown to have a dose-dependent depigmenting activity in vivo, with no irritation or other side-effects.

By studying the ultrastructural changes in melanosomes and their transfer following RWJ-50353 treatment, we identified the accumulation of melanosomes within treated melanocytes, with an increase in early stages and empty melanosomes. We also identified an increase in melanosome-containing dendrites within

(f)
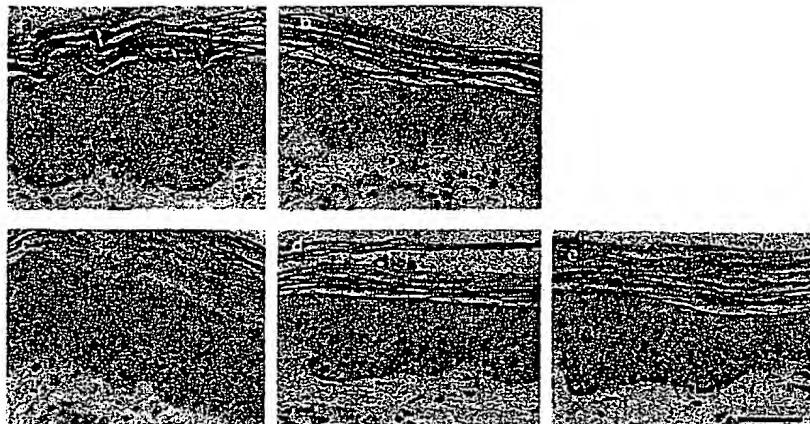
Figure 6. The depigmenting effect of RWJ-50353 is reversible. Yucatan swine were treated for 8 wk with 250 μM of RWJ-50353, and followed without treatment for a further 4 wk. Biopsies were taken before the start of treatment (a), after 8 wk (completion of treatment phase, b), and at the ninth, tenth, and twelfth weeks (c–e, 1–4 wk after treatment was terminated). Scale bar: 12 μm. F&M-stained sections revealed re-pigmentation after treatment had been stopped, with no irritation or other side-effects. Differences in epidermal thickness result from the different sites of biopsies, and have no correlation to treatments. (f) Image analysis of F&M-stained sections was used to quantify pigmentation, relative to the untreated control.
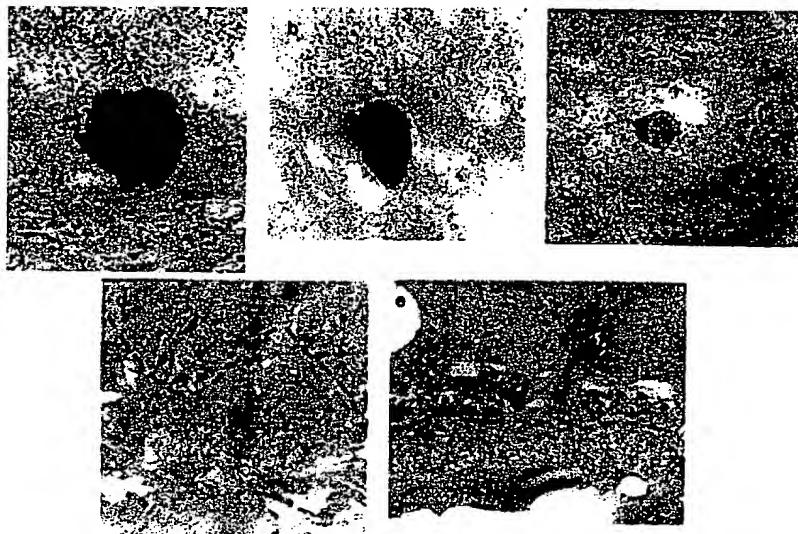

Figure 7. Electron micrpscopy analysis of Yucatan swine skin treated with RWJ-50353. (a) A representative melanosome inside a keratinocyte of untreated swine skin. (b), (c) Representative melanosomes inside keratinocytes of RWJ-50353 (250 μM) treated swine skin are smaller and less pigmented than control ones. (d) A random distribution of melanosomes in control swine epidermis. (e) Melanosomes of RWJ-50353 (250 μM) treated swine skin are detected mainly at the epidermal–dermal border (marked). Scale bar: (a–c) 0.05 μm; (d, e) 0.8 μm.

treated keratinocytes. Although we cannot rule out other mechanisms, we suggest that neither melanosome formation and function nor dendrite penetration into the keratinocytes are directly affected by the drug. We propose that RWJ-50353-treated keratinocytes are unable to actively take or receive melanosomes from the presenting dendrites. This keratinocyte "inability" leads to the accumulation of melanosomes in the melanocytes, and could possibly turn on a negative feedback mechanism that slows pigment production. Our earlier data document reduced TRP-1 expression following RWJ-50353 treatment (Seiberg et al, 2000). Such a negative feedback mechanism could explain the reduced TRP-1 expression, as TRP-1 is a major melanosomal glycoprotein. Following RWJ-50353 treatment the melanocytes contain more melanosomes than required for homeostasis, and therefore melanosome production, and TRP-1 synthesis, are reduced. The increase in pigment deposition within melanocytes following PAR-2 activation cannot be mechanistically explained by melanosome trafficking only, and requires further study.

In vivo ultrastructural studies revealed an abnormal distribution of melanosomes in RWJ-50353-treated swine skin. The polarity of this distribution, at the dermal-epidermal border, could provide a clue to extracellular matrix components or adhesion molecules

involved in melanosome transfer. It is not likely that melanosomes were released into the intercellular space, and were not taken immediately by keratinocytes, as such a mechanism should result in melanosome accumulation and skin darkening, which were not observed. Therefore, we assume that these melanosomes were later translocated into the keratinocyte using either a PAR-2-independent mechanism or a re-activated PAR-2. As in vivo we could not completely inhibit melanogenesis or pigment transfer with RWJ-50353 (up to 10 mM, twice daily treatment), we suggest that either the keratinocyte PAR-2 is not the only mechanism for melanosome transfer, or that PAR-2 is re-activated when RWJ-50353 levels are reduced. It is important to note that in RWJ-50353-treated skin the transferred melanosomes are of poor quality, reflecting changes in melanosome formation and/or melanogenesis prior to their transfer. These changes agree with our suggested negative feedback mechanism, by responding to accumulation of nontransferred melanosomes with reduced new pigment production. Although the drug indirectly affected melanosome quality, it had no effect on the mode of melanosome transfer. As expected for dark skinned individuals, the dark skinned swine melanosomes were always transferred singly, and melanosome complexes were never observed, regardless of the treatment.

The epidermal-melanin unit, a functional unit that produces and distributes melanin (reviewed in Ortonne, 1995), is shown to have a role in the regulation of pigmentation. The keratinocyte PAR-2 is involved in the regulation of melanosome transfer, and therefore affects skin pigmentation. Modulation of the PAR-2 pathway with serine protease inhibitors such as RWJ-50353 could offer an alternative to depigmenting therapies.

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SUBCELLULAR LOCALIZATION OF MELANIN BIOSYNTHESIS

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Tyrosinase from different sources has different characteristics;[1] that obtained from plant tissue can usually be prepared in colloidal solution, while that obtained from mammalian tissue is held by ultramicroscopic, cytoplasmic particles. Localization of tyrosinase activity in suspensions of cell particles was first reported by Herrmann and Boss in 1945[2] in preparations of the ciliary body of the bovine eye. Later, in 1949, Lerner et al.[3] also reported that in their differential-centrifugation studies of suspensions of homogenized Harding-Passey mouse melanoma, tyrosinase activity was found in the particulate fraction. They conjectured that the structural elements of this fraction were "microsomes, or particles the size of microsomes, which were formerly part of larger aggregates that were dispersed during the experiments." The nature of the particle to which tyrosinase is attached remained a mystery for the next ten years.

Studies made with the electron microscope have led to the belief that melanin granules and mitochondria are distinct cytoplasmic particles,[4-6] while biochemical assays and vital staining provided data suggesting that the melanized cytoplasmic particles present in suspensions of mouse melanoma are modified mitochondria.[7,8] The need for resolution of these differences of opinion has been emphasized by Dalton[9] and Birbeck and Barnicot[10] who make a plea for correlation of the findings in electronmicrographs with chemical data obtained from melanin granules and mitochondria after they have been separated. Such a study has been carried out with experimental procedures that provide a good separation of melanin granules and mitochondria.

The experiments to be presented here provide conclusive data in support of the concept that melanin granules and mitochondria are distinctive subcellular particles in the mammalian melanocyte.

Observations made by light and electron microscopy have led to the development of three major theories about the origin of melanin granules: the theory of *nuclear origin*,[11,12] according to which granules are supposed to be composed of extruded nuclear material; the theory of *mitochondrial origin*,[7,8,13] which has just been briefly outlined; and the theory of *Golgi origin*,[4,14,15] which postulates that granules are formed in the Golgi apparatus during the early stages of their

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development. Wissenfels has suggested still another theory according to which prepigment granules are formed at multiple sites (*Pigmentbildungzentrum*) in the cytoplasm.[16]

Most investigations of this problem have been largely morphological. Biochemical data are needed, however, for definition of the dynamic, metabolic pathways involved in the formation of melanin granules. An investigation is reported here in which ultracentrifugal separation of cell particles, incorporation of radioactive tracers and electromicroscopy have all been employed to reveal the steps which may be involved in the formation of melanin granules. Preliminary reports of this work have already been published within the last few years.[17],[18] Subsequent biochemical findings now justify an expansion of these concepts. Experimental data obtained in this series of studies are all compatible with the hypothesis that tyrosinase is synthesized in "small granules," which are presumably ribonucleoprotein particles, and subsequently transfered to and stored in melanosomes.

Terminology of Specialized Organelles in Melanocytes

Studies of the fine structure of the melanocyte have in the last four years altered the concept of the nature of this cell. Barnicot and Birbeck[4] were the first to point out that melanocytes contain a system of membranes similar to those seen in secretory cells. In addition, they have observed that melanin granules in the cytoplasm of the melanocyte are not of uniform structure, but appear to exist in varying stages of development; the granules start as "hollow vacuoles in which a tenuous material appears in the form of a folded lamella ... the material is rapidly thickened and defined by the deposition of more dense material, until a solid body about twice the size of the original vacuole is formed." Subsequent studies of the fine structure of melanin granules[6,14,16,19] have confirmed and extended these findings. In the study reported here, the particles isolated from pigmented tissues by ultracentrifugal, density-gradient methods have been examined by both biochemical techniques and electronmicroscopy. The results obtained have made it possible to outline a pathway of development of melanin granules. Because melanin granules are specialized organelles which differ from mitochondria not only in structure and development, but also in chemical and enzymic composition, a set of new terms is proposed for the various stages of their development. Justification for this introduction of new terms is the inadequacy of the single, inclusive term, "melanin granule," to describe with precision the structural and chemical metamorphoses which take place in the granule during melanization. In FIGURE 1 have been outlined the essential features of premelanosomes, melanosomes, and melanin granules: the three specialized organelles found in melanocytes.

It is believed that three stages can be recognized in the formation of the *melanin granule*: the final product of the melanocyte (FIGURE 1):

I. A first stage in which polypeptides are synthesized and condensed into the secondary and tertiary structures ("*pro-tyrosinase*") of the enzymic protein molecules.

II. An intermediate stage in which these "pro-tyrosinase" molecules are

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arranged in ordered structures, *premelanosomes* (the enzyme molecules of "pro-tyrosinase" are aligned in an ordered pattern).

III. A third stage in which melanin biosynthesis begins and melanin accumulates inside the premelanosome. During this stage, the granule contains tyrosinase activity and is a *melanosome.*

The final product, in which melanization is complete, and in which no tyrosinase may be detected, is a *melanin granule.*

During Stage II, the arrangement, shape and size of the premelanosomes are thought to be both species- and site-dependent. Melanin is not yet syn-

NOMENCLATURE	MORPHOLOGY	BIOCHEMICAL COMPOSITION		
RIBOSOME	• 100-150 Å	RNA + PROTEIN	Site of polypeptide biosynthesis	I
GOLGI VESICLE	o 0.05μ	PHOSPHOLIPID + PROTEIN	Condensation (?)	
INTERMEDIATE VESICLE	0.5μ	PHOSPHOLIPID + PROTEIN	Stage of "Pro-Tyrosinase" arrangement in a structural form	II
PREMELANOSOME	0.7 x 0.3μ*	"PRO-TYROSINASE"	End stage of "Pro-Tyrosinase" arrangement and final product in albino melanocyte	
MELANOSOME	0.7 x 0.3μ*	TYROSINASE + MELANIN	Specific site of melanin formation	III
MELANIN GRANULE	0.7 x 0.3μ*	MELANIN + NO MEASURABLE TYROSINASE ACTIVITY	Final product of melanocyte	

* *Typical values for human brown melanin granules.*

FIGURE 1. Stages in the development of melanin granules: I, biosynthesis of protein; II, biosynthesis of organelle; III, biosynthesis of melanin.

thesized at this stage. During the early parts of Stage III, the lamellar pattern of the premelanosome can still be seen in electronmicrographs, but as the process of melanization continues, the lamellar pattern gradually becomes obliterated by deposited melanin and an amorphous *melanin granule* eventually results. As melanin gradually forms within the melanosome, the tyrosinase activity of the melanosome correspondingly decreases: in other words, melanin formation and tyrosinase activity are inversely related.[20]

Although melanin is synthesized *in vivo* only during Stage III, active tyrosinase may be recovered *in vitro* from the ribonucleoprotein particles. It is presumed therefore that extraction of the enzymic protein results in its activation. There is reported to exist in insects an inactive enzyme called "pro-

tyrosinase" which may be activated both *in vivo* and *in vitro*.[21-24] The term "*pro-tyrosinase*" is here tentatively applied to the enzymic protein of the melanocyte during Stages I and II when the enzyme molecules are thought to have been synthesized but not yet aligned for catalytic activity.

Materials and Methods

Preparation of the Various Cell Components

B-16 mouse melanoma was serially transplanted in C-57 strain mice. The entire, actively growing melanoma was excised when it reached 1.0 to 1.5 cm. in diameter and was promptly homogenized in 0.25 M sucrose at about 0° C. All subsequent processing (see TABLE 1) took place in a cold environment (about 3° C.). The nuclear fraction was prepared by centrifuging the homogenate for 10 min. at 700 g. The resulting low-speed supernatant, when centrifuged at 11,000 g for 10 min., yielded a sediment that was resuspended in 0.25 M sucrose and recentrifuged at 15,000 g for 10 min. This sediment (the large-granule fraction) was again suspended in 0.25 M sucrose to make the large-granule preparation. Small-granule fraction No. 1 was prepared by centrifuging the washes and the supernatant from the large-granule fraction in the Spinco ultracentrifuge for 60 min. at 105,000 g. The resulting supernatant is hereafter referred to as the soluble fraction. Small-granule fraction No. 1 was resuspended in 0.25 M sucrose solution (usually 1:1/v:wet weight) to make small-granule preparation No. 1 (FIGURE 2). For some experiments, the method of preparing the small-granule fraction was modified as follows: the low-speed supernatant obtained after centrifugation at 700 g for 10 min. was recentrifuged at 21,000 g for 10 min. The resulting supernatant and washes were then recentrifuged in the Spinco ultracentrifuge described above. The sediment thus obtained constitutes what is here called small-granule fraction No. 2. When small-granule fraction No. 2 is resuspended in 0.25 M sucrose solution, it constitutes small-granule preparation No. 2.

The Nuclear Fraction

Isolation of nuclei. The method used for isolation of nuclei from the so-called "nuclear fraction" is a modification of that described by Allfrey *et al.*[25] All procedures were carried out in the cold (between 2 and 4° C.). The nuclear fraction obtained from the homogenate prepared from 30 gm. of tumor tissue was gently homogenized again in the blendor at 1,000 rpm for 10 min. The resulting suspension was filtered through a double layer of gauze (Johnson & Johnson, type 1) and then through a single thickness of double-napped flannelette. The filtrate was centrifuged at 700 g for 7 min. and the supernatant discarded. The sediment was resuspended in 100 ml. of 0.25 M sucrose-0.003 M CaCl₂ solution and again passed through the flannelette. The filtrate thus prepared was centrifuged at 700 g for 5 min. and the resulting sediment, which contained the nuclei, was washed by centrifugation in 100 ml.-amounts of 0.25 M sucrose-0.003 M CaCl₂ which were changed repeatedly until the supernate became water-clear. The final sediment was suspended in 10 ml. of 0.25 M sucrose-0.003 M CaCl₂ to form the purified preparation of nuclei.

oth *in vivo* and *in vitro*.[21-24] The term
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ND METHODS

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. by centrifugation in 100 ml.-amounts
:h were changed repeatedly until the
al sediment was suspended in 10 ml.
m the purified preparation of nuclei.

Preparation of the Density Gradients

Density gradients were prepared in the tubes of the Spinco swinging-bucket
rotor (SW-39-L) by layering 0.5 ml. of 8 different concentrations of sucrose
solution in serial order, with the most concentrated layer at the bottom of the
tube. They were then allowed to stand for between 12 and 20 hours so that

TABLE 1
PREPARATION OF CELL COMPONENTS
All procedures were carried out in a cold environment (0° to 4° C.)

TISSUE

(In 5–9 vols. of isotonic sucrose solution)

homogenized for 2 min.
(Potter-Elvehjem type homogenizer)

centrifuged
700 g for 10 min.

Sediment——————————————Supernatant
NUCLEAR FRACTION
(may contain erythro-   centrifuged
cytes, connective tissue,   11,000 g for 10 min.
residual intact cells)

Sediment————————————Supernatant*

resuspended

centrifuged
15,000 g for 10 min.

Sediment——————————Supernatant
LARGE-GRANULE
FRACTION   mixed with
                               Supernatant*——————→
resuspended in
isotonic sucrose

LARGE-GRANULE   centrifuged
PREPARATION   105,000 g for
(melanosomes)   60 min.

Sediment————————————Supernatant
SMALL-GRANULE   SOLUBLE
FRACTION No. 1   FRACTION

resuspended in
isotonic sucrose

SMALL-GRANULE
PREPARATION No. 1

* Supernatants marked with an asterisk were mixed before the final centrifugation.

the gradient might become smooth. At the end of this interval, 1 ml. of freshly prepared granule preparation was layered carefully over the top of each tube in its respective series: *i.e.*, large-granule series or small-granule
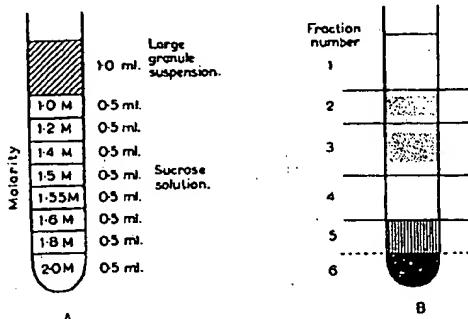

FIGURE 2. (A) Density-gradient tube before centrifugation, showing the layering of eight different concentrations of sucrose solution and the large-granule preparation (in 0.25 *M* sucrose). The gradient was prepared 18 hours in advance; the large-granule preparation was added immediately before centrifugation. (B) Fractions 1 through 6 as they appear in the gradient tube after ultracentrifugation at 103,000 g for 1 hour in a horizontal rotor. The four opaque fractions and the places where the tube was cut are shown diagrammatically.
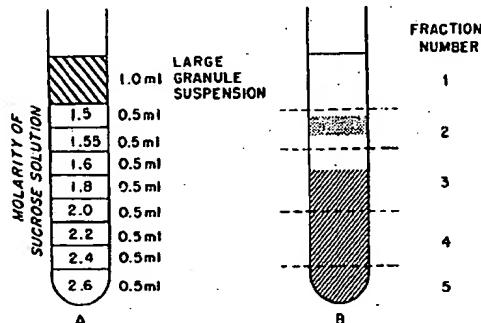
FIGURE 3. (A) Tube containing a modified density gradient with large-granule preparation before centrifugation; the gradient consists of 8 different concentrations of sucrose solution, and was prepared 18 hours previous to centrifugation. The large-granule preparation in 0.25 *M* sucrose was added immediately before centrifugation. (B) Fractions 1 through 5 as they appear in the gradient tube after ultracentrifugation at 103,000 g for 1 hour. The 2 opaque regions and the places where the tube was cut are shown diagrammatically.

series. The sucrose concentrations used for study of the large-granule preparation in the *low-density* studies were 2.0 *M*, 1.8 *M*, 1.6 *M*, 1.55 *M*, 1.5 *M*, 1.4 *M*, 1.2 *M*, 1.0 *M* (FIGURE 2); in the *high-density* series they were 2.6 *M*, 2.4 *M*, 2.2 *M*, 2.0 *M*, 1.8 *M*, 1.6 *M*, 1.55 *M*, and 1.5 *M* (FIGURE 3). For analysis of the small-granule preparation, the sucrose concentrations were 1.8 *M*, 1.6 *M*, 1.4 *M*, 1.2 *M*, 1.0 *M*, 0.8 *M*, 0.6 *M*, and 0.4 *M* (FIGURE 4).

1. At the end of this interval, 1 ml. of
)n was layered carefully over the top of
i.e., large-granule series or small-granule

Fraction number

1
2
3
4
5
6

B

...efore centrifugation, showing the layering of eight
)n and the large-granule preparation (in 0.25 M
hours in advance; the large-granule preparation
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E
ULE
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FRACTION NUMBER

1
2
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B

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...sed for study of the large-granule prepara-
.0 M, 1.8 M, 1.6 M, 1.55 M, 1.5 M, 1.4 M,
gh-density series they were 2.6 M, 2.4 M,
', and 1.5 M (FIGURE 3). For analysis of
ucrose concentrations were 1.8 M, 1.6 M,
and 0.4 M (FIGURE 4).

Separation of the Various Fractions after Centrifugation

At the end of centrifugation, after the position and thickness of the various strata in each tube had been recorded (FIGURES 2, 3, 4), the strata (fractions) were separated by means of a specially designed centrifuge-tube cutter.[13] FIGURES 2, 3, and 4 show where the tube was cut to permit collection of the individual fractions and how the fractions were numbered.

Enzyme Assays

Determinations of succinoxidase and glutamate-oxidase content were made immediately after separation of the fractions; determinations of tyrosinase and protein-nitrogen content were always completed within 48 hours after separation.

MOLARITY OF SUCROSE SOLUTION

1.0 ml
0.4 — 0.5 ml
0.6 — 0.5 ml
0.8 — 0.5 ml
1.0 — 0.5 ml
1.2 — 0.5 ml
1.4 — 0.5 ml
1.6 — 0.5 ml
1.8 — 0.5 ml

SMALL GRANULE SUSPENSION

FRACTION NUMBER

1
2
3
4
5

A   B
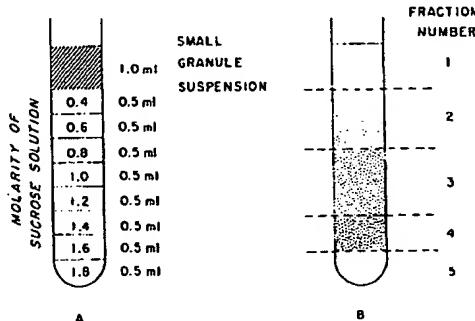
FIGURE 4. (A) Density-gradient tube before centrifugation, showing the 8 different concentrations of sucrose solution and the preparation of small granules in 0.25 M sucrose. The gradient was prepared 18 hours in advance; the small-granule preparation was added immediately before centrifugation. (B) Fractions 1 through 5 as they appear in the gradient tube after ultracentrifugation at 103,000 g for 1 hour in a horizontal rotor. The 2 opaque fractions and the places where the tube was cut are shown diagrammatically.

The *succinoxidase* content of the fractions was estimated respirometrically by measuring oxygen consumption in the presence of cytochrome-C, as described by Umbreit et al.;[24] some estimations were also made with ATP (2.8×10^{-3} M) and DPN (1.5×10^4 M) as cofactors.

Glutamate oxidase was estimated respirometrically by measuring oxygen uptake in the following reaction system: potassium phosphate buffer, pH 7.5 (1.2×10^{-2} M); ATP (2.5×10^{-3} M); DPN (1.5×10^4 M); nicotinamide (1.2×10^{-2} M); $MgCl_2$ (8×10^{-3}); cytochrome-C (8.3×10^{-6}); and glutamic acid (1×10^{-2} M) in the presence of KOH in the center well.

Tyrosinase content was estimated respirometrically by measuring oxygen consumption, using a 10:1 mixture of L-tyrosine and L-dopa as substrate (1.77 μmole) in the M/10 phosphate buffer (pH 6.8).[27]

Biochemical Analysis

The samples were analyzed by the technique of Schmidt and Thannhauser,[29] modified by the substitution of perchloric acid for trichloroacetic acid. The

phospholipid content was estimated from the phosphorous content of the lipid fraction. Ribonucleic acid and deoxyribonucleic acid content was calculated from the phosphorous content of the RNA fraction (RNA-P) and the DNA fraction (DNA-P). To determine the protein-nitrogen content, trichloroacetic acid was added to each sample to precipitate the protein, the precipitate was spun down, washed once with trichloroacetic acid and dissolved in 1 N NaOH solution. The nitrogen content of this alkaline solution was determined by the method described by Johnson in 1941.[29]

Morphological Methods

For electronmicroscopy, suitable aliquots of each fraction were mixed at 0° C. with 2 per cent osmium tetraoxide buffered with acetate veronal to pH 7.4 and centrifuged at 105,000 g for 1 hour. The sediment obtained was then dehydrated in a graded series of ethyl alcohols, embedded in araldite resin, sectioned with a modified Cambridge rocker-microtome and examined by means of a Siemans Elmiskop I.

In Vivo Incorporation of Leucine-C[14] by Soluble Tyrosinase

Treatment of animals. Eight or ten adult, female mice bearing B-16 melanomas 1-2 cm. in diameter were given an intraperitoneal injection of 1 μc./10 gm. body weight of uniformly labeled L-leucine-C[14] (Nuclear Chicago) in 0.05 ml. of buffered saline solution.

Cell fractionation procedure. The mice were killed at various intervals after injection of the leucine-C[14], and the melanoma was immediately excised and homogenized in 5-9 volumes of ice-cold 0.25 M sucrose solution. The homogenate was separated into fractions by differential centrifugation at 0° C. by a modification of the procedure of van Lancker and Holtzer.[30]

The supernatant resulting from centrifugation of the homogenate at 700 g for 10 min., when recentrifuged at 11,000 g for 10 min., yielded a sediment which was resuspended in 0.25 M sucrose and recentrifuged at 11,000 g for 10 min. This sediment, the large-granule fraction, was used as one of the starting materials for the extraction of soluble tyrosinase. The supernatant obtained after the second centrifugation (11,000 g for 10 min.) was recentrifuged at 25,000 g for 10 min. and the resulting supernatant was subjected to further high-speed centrifugation at 105,000 g for 1 hour to yield the small-granule fraction.

Extraction of Soluble Tyrosinase

Soluble tyrosinase was extracted from both the small-granule fraction and the large-granule fraction by a modification of the procedure of Brown *et al.*[31] The fraction to be tested was suspended in 30 ml. of cold, 0.5 per cent sodium deoxycholate solution in 0.05 M tris buffer at pH 8.0 and homogenized for 1 min. The resulting suspension was then one tenth saturated with $(NH_4)_2SO_4$ by the addition of saturated $(NH_4)_2SO_4$, mixed with an equal volume of cold (3° C.) acetone, and filtered through a Buchner funnel at room temperature (22° C.) to remove the precipitate. Another volume of cold acetone was added and the mixture was centrifuged. The precipitate obtained was resuspended

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·gical Methods

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·ucine-C¹⁴ by Soluble Tyrosinase

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· differential centrifugation at 0° C. by a
Lancker and Holtzer.[30]
ntrifugation of the homogenate at 700 g
1,000 g for 10 min., yielded a sediment
rose and recentrifuged at 11,000 g for 10
le fraction, was used as one of the starting
e tyrosinase. The supernatant obtained
)00 g for 10 min.) was recentrifuged at
ng supernatant was subjected to further
g for 1 hour to yield the small-granule

Soluble Tyrosinase

rom both the small-granule fraction and
ication of the procedure of Brown *et al.*[31]
led in 30 ml. of cold, 0.5 per cent sodium
buffer at *p*H 8.0 and homogenized for 1
hen one tenth saturated with (NH₄)₂SO₄
.O₄, mixed with an equal volume of cold
a Buchner funnel at room temperature
.nother volume of cold acetone was added
he precipitate obtained was resuspended

in 5 ml. of 0.1 *M* phosphate buffer at *p*H 6.8 and dialyzed against 5 l. of cold,
distilled water overnight (18 hours) with at least 2 changes of distilled water.
The dialyzed suspension was made up to 7 ml. by the addition of 0.1 *M* phos-
phate buffer at *p*H 6.8 and centrifuged to separate out the clear supernatant
which contained the soluble tyrosinase.

Agar Electrophoresis

Agar electrophoresis (K. Shimao, unpublished method) was carried out at
4° C. with 0.05 *M* veronal buffer at *p*H 8.6 on agar gel 1 mm. thick. The 1 mm.
film of agar gel was prepared by pouring 6.5 ml. of hot 1 per cent agar solution
into a plastic frame (inner dimension 70 X 90 mm.) which was clipped to a glass
plate. After the agar had been chilled for 30 min., a 2 X 6 mm. strip of What-
man No. 3 MM filter paper was moistened with about 10 *μ*l. of the soluble
tyrosinase solution to be analyzed and placed on the center of the agar plate.
The gel was connected to the voltage supply by a strip of buffer-moistened
filter paper and a platinum electrode that were both dipped in a vessel of buffer.
After covering the gel with a glass plate, electrophoretic migration was allowed
to continue for 4 to 5 hours with a current of 8 mAmp. Parallel tests were
always made simultaneously with normal human serum to provide a reference
of mobility.

To demonstrate the site of tyrosinase activity in the gel, a sheet of Whatman
No. 3 MM filter paper the same size as the gel plate was moistened with 0.33
mg./ml. L-dopa in 0.1 *M* phosphate buffer at *p*H 6.8 and superposed on the gel.
After standing at room temperature for 10 to 20 min., the site of tyrosinase
activity could be detected by appearance of the orange color of dopachrome
or the purple color of melanin.

Protein was stained by dipping the gel in a 0.5 per cent solution of aniline
blue-black in a mixture of ethanol and glacial acetic acid (9:1/v:v). The
excess dye was washed out with 5 per cent acetic acid.

Measurement of the Radioactivity of the Soluble Tyrosinase

A 3-ml. aliquot of the soluble tyrosinase was mixed with an equal volume
of 10 per cent trichloroacetic acid and heated to 95° C. for 15 min. The pro-
tein thus precipitated was collected by centrifugation and washed again with
hot, 5 per cent trichloroacetic acid; it was then subjected to 2 washings each
with an alcohol-ether mixture at 45° C. (3:1/v:v) and ether at 38° C. The
final precipitate was dried by heating to 50° C. and dissolved in 1 ml. of 1 *M*
hydroxide of Hyamine 10-X (P-(di-isobutyl-cresoxyethyl)dimethyl-benzyl-
ammonium hydroxide) in methanol.[32] This solution was transferred quanti-
tatively to a vial by three 5-ml. washings of scintillating solution (4 gm. of 2-5-
diphenyloxazole and 100 mg. of 1,4-bis-2-(phenyloxazole)-benzene dissolved
in 1000 ml. of toluene) and counted in the Packard liquid-scintillation counter
at least 3 times. A known amount (2540 d.p.m.* in 0.5 ml. of toluene) of
benzoic-C¹⁴ acid was then added to the vial and the mixture was counted again
in the same counter. The d.p.m. value of the samples was calculated from
the difference between the counting rate with and without the internal standard

* Disintegration per minute.

and the counting rate without the internal standard. Ether-washed, dry protein was obtained in the same way from another aliquot (2 ml.) of the soluble tyrosinase and dissolved in 0.1 N sodium hydroxide so that its nitrogen content could be determined by the method already described. The specific radioactivity of the soluble tyrosinase is expressed as d.p.m./μg. protein-nitrogen.

The radioactivity of components isolated from soluble tyrosinase by means of agar electrophoresis was determined as follows. At the end of the period of electrophoretic migration, the areas in which tyrosinase activity and protein were present were demonstrated by means of the dopa reaction and acid blue-black, respectively. After the agar plate had been allowed to dry thoroughly at room temperature, the parts of the agar where the dopa reaction was positive and the parts where the dopa reaction was negative, but the acid blue-black stain positive were scraped from the agar plate with a scalpel and suspended in 15 ml. of scintillating solution. The radioactivity of this material was counted as described above.

TABLE 2

DISTRIBUTION OF TYROSINASE ACTIVITY IN THE VARIOUS FRACTIONS ISOLATED FROM B-16 MOUSE MELANOMA

Fraction	Tyrosinase activity (μl. O₂/min.)	Recovery (%)
Nuclear	6.3	28
Large granule	9.55	42.4
Small granule	4.2	18.6
Soluble	2.49	11.0

RESULTS

Distribution of Tyrosinase Activity in the Various Fractions

The various fractions of mouse melanoma were isolated by the differential centrifugation described above and made up to the same volume as the homogenate which was used as starting material after 0.25 M sucrose solution had been added. TABLE 2 shows the distribution of tyrosinase activity in the various fractions.

NUCLEAR FRACTION

The nuclear fraction studied in this series of experiments contained intact tumor cells, connective tissue, red blood cells, cell debris, and approximately one third of the total amount of tyrosinase activity recovered from the homogenized melanoma tissue. It seemed worthwhile, therefore, to try to determine whether or not any tyrosinase activity was present in the nuclei themselves. By a slight modification of the biochemical procedures described by Allfrey et al.[25] it was possible to prepare from the so-called nuclear fraction suspensions which, when examined under the light microscope, were seen without stain to contain great numbers of free nuclei, a few whole blood cells, clumps of brown pigment granules, and occasional red blood cells. When

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The radioactivity of this material was

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THE VARIOUS FRACTIONS ISOLATED FROM B-16
MELANOMA

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these suspensions were stained with hematoxylin-eosin, occasional strands of
cytoplasm and some brown pigment granules could be seen, as well as bits of
amorphous connective tissue stained with eosin. It is estimated that nuclei,
per se, constituted somewhat more than 70 per cent of the suspended material.*

The final, purified preparation of nuclei contained 22.8 per cent of the total
amount of DNA and 4.3 per cent of the total tyrosinase activity of the nuclear
fraction. Assuming that isolation of the purified nuclei has been accomplished
without significant loss of DNA, the tyrosinase activity within this purified
preparation may be considered to be 18.9 per cent of the initial activity of the
nuclear fraction. Using as a basis for calculation the experimentally obtained
figure of 28 per cent recovery of tyrosinase activity in the nuclear fraction
(TABLE 2), it appears that one might expect to recover from the purified prepara-
tion of nuclei 5.3 per cent of the total tyrosinase activity of whole tissue.

LARGE-GRANULE FRACTION

Separation by density-gradient centrifugation. When the density-gradient
shown in FIGURE 2 was centrifuged with the large-granule preparation at
103,000 g for 1 hour in a horizontal rotor, 2 narrow bands typically formed in
the contents of the tube, one (Fraction 2) at the top of the gradient, the other
(Fraction 3) a gray, turbid layer that contrasted with the relatively clear zone
below (Fraction 4). Near the bottom of the tube, there was a suspension of
black particles (Fraction 5) and a black pellet (Fraction 6).

Distribution of tyrosinase, succinoxidase, and protein-nitrogen. The tyrosinase
activity, succinoxidase activity and protein-nitrogen content of all 6 fractions
were determined by the method described above. Percentage, as shown in the
various figures, is calculated in terms of the sum of the amounts of material
recovered in all fractions. FIGURE 5 shows the estimates of succinoxidase and
tyrosinase activity and protein-nitrogen content in B-16 mouse melanoma.
Each point on the curve is the average of 6 observations. The curve for
succinoxidase activity reached its peak in Fraction 3, which contained 70 per
cent of the sum of the activity recovered in all fractions. Some succinoxidase
activity was evident in Fractions 4, 5, and 6. Fraction 6 also contained tyro-
sinase activity: 94 per cent of the amount contained in Fractions 1 through 6.

Electronmicrographs show that the large-granule preparation contains many
cell elements, such as cell debris, mitochondria, microsomes, endoplasmic re-
ticulum, and melanosomes (FIGURES 6 and 8–12). In the melanosomes, a single
outer membrane and an internal membrane composed of irregular, crumpled,
spiral, and parallel strands in various stages of melanization can be recognized.
Fraction 2 consists mainly of cell debris, and Fraction 3 is an almost pure
preparation of mitochondria (FIGURES 9 and 10). Fraction 5 contains both
mitochondria and melanosomes, and Fraction 6 is almost exclusively melano-
somes (FIGURES 11 and 12). It is not possible to prepare really good electron-

* Under the light microscope, counts were made of the number of nuclei, intact cells, clumps
of brown pigment granules, pieces of amorphous connective tissue, and red blood cells present
in stained smears of the purified suspension of nuclei. The average of counts obtained from
25 fields chosen at random was as follows: nuclei, 1198; intact cells, 59; clumps of brown pig-
ment granules, 64; bits of connective tissue, 207; red blood cells, 36. From these figures,
the purity of the suspension of nuclei may readily be calculated.

micrographs, particularly of Fractions 5 and 6, because sucrose in high concentration tends to distort some structures (e.g., mitochondria) osmotically and because the hard melanosomes make Fractions 5 and 6 difficult to cut. The micrograms obtained were sufficiently clear, however, to permit adequate identification of the various elements present.

In order to separate mitochondria satisfactorily from melanosomes, the density gradient shown in FIGURE 3 was prepared with sucrose and the large-granule preparation. The typical appearance of the tube after centrifugation at 103,000 g for 1 hour in a horizontal rotor is shown in FIGURE 3; a narrow,
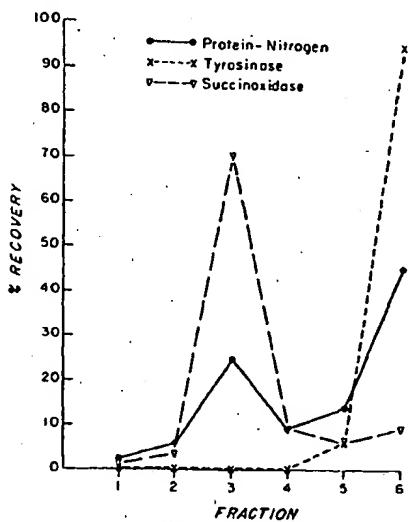

FIGURE 5. Recovery of protein-nitrogen, tyrosinase, and succinoxidase in Fractions 1 through 6 of large-granule preparation of B-16 mouse melanoma following density-gradient centrifugation. Percentages are calculated in terms of the sum of the amounts recovered in all fractions. Each point on the curve is the average of six observations. The various fractions are numbered as in FIGURE 2. Total recoveries were: protein-nitrogen, 97.8 per cent ± 1.26; tyrosinase, 293 per cent ± 29.3; succinoxidase, 80.5 per cent ± 2.4.

brownish-gray, relatively tightly packed band (Fraction 2) can be seen at the top of the gradient. There is a relatively clear zone between this layer and the brown or black suspension that fills almost the entire bottom half of the tube.

FIGURE 6 shows the typical distribution obtained with the high-density gradient. In this experiment (see FIGURE 7), glutamate oxidase activity and also succinoxidase activity were determined in fractions separated by high density-gradient centrifugation. Here the peaks of both succinoxidase and glutamate oxidase activity were found in Fraction 2, which corresponds to Fractions 2 and 3 in the low density-gradient experiments. No succinoxidase activity was found in Fractions 4 and 5. Tyrosinase activity appeared only in Fractions 4 and 5. The detailed results of this experiment (TABLE 3) show that

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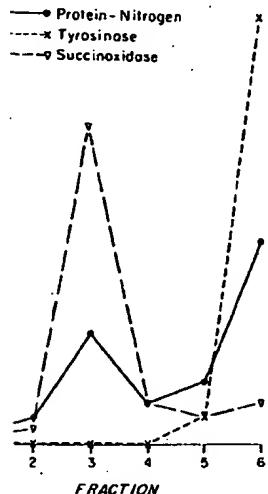
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Fraction 5 (melanosomes) contains 3 times as much tyrosinase per milligram of
protein-nitrogen as the unfractionated large-granule preparation and that Frac-
tion 2 (mitochondria) contains twice as much succinoxidase and 3 times as much

FIGURE 6. Fraction 6 isolated from the large-granule preparation of B-16 mouse mela-
noma by density-gradient centrifugation (see FIGURE 12) in more detail. The varied size
and degree of melanization of the particles can be seen. ×80,000.

glutamate oxidase per milligram of protein-nitrogen as does the uncentrifuged
large-granule preparation.

SMALL-GRANULE FRACTION

Separation by density-gradient centrifugation. When the density-gradient
shown in FIGURE 4 was centrifuged with small-granule preparation No. 1

at 103,000 g for 1 hour in a horizontal rotor, a relatively wide, pinkish-red, clear band (Fraction 2) typically formed in the contents of the tube below the clear top layer (Fraction 1). A wide zone of opalescent material without any sharp lower boundary extended from the bottom of Fraction 2 down to the level in the tube where 1.6 M sucrose was superimposed upon 1.8 M sucrose when the solutions were originally layered (FIGURE 4). For sampling, the opalescent zone was divided into two parts, Fractions 3 and 4. The translucent and colorless zone at the bottom of the tube below Fraction 4 is Fraction 5; it contains a few black pellets. When small-granule preparation No. 2 was subjected to density-gradient analysis, 5 fractions were
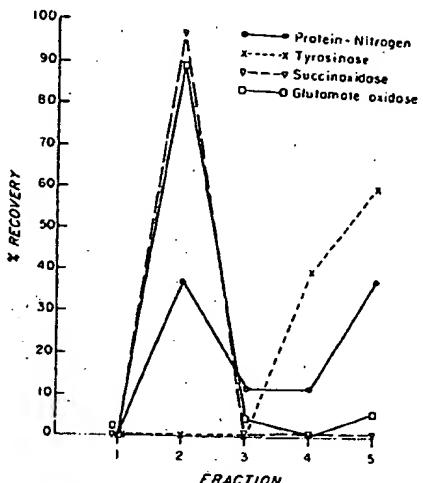
FIGURE 7. Recovery of protein-nitrogen, tyrosinase, succinoxidase, and glutamate oxidase in Fractions 1 through 5 of large-granule preparation of B-16 mouse melanoma following density-gradient ultracentrifugation. Percentages are calculated in terms of the sum of the amounts recovered in all fractions. Total recoveries were: protein-nitrogen, 94.3; tyrosinase, 417 per cent; succinoxidase, 82 per cent; and glutamate oxidase, 124 per cent.

obtained exactly like those of small-granule preparation No. 1, except that there was no precipitate in Fraction 5 at the bottom of the tube.

Distribution of tyrosinase, RNA, phospholipid, and protein in the various fractions. All five fractions were examined for tyrosinase activity, RNA, phospholipid, and protein content by the method described above. FIGURE 13 shows the distribution of tyrosinase activity in the various fractions derived from small-granule preparation No. 1. The curve for tyrosinase activity had 2 peaks, one in Fraction 5, which contained 56.6 per cent of the total activity recovered in all fractions, the other in Fraction 2, which contained 23 per cent of the total activity. Since Fraction 5, isolated from small-granule preparation No. 1 by density-gradient centrifugation, was found by electronmicroscopy to contain melanosomes, the greater centrifugal force of 21,000 g was needed

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Electronmicrographs of the Various Fractions

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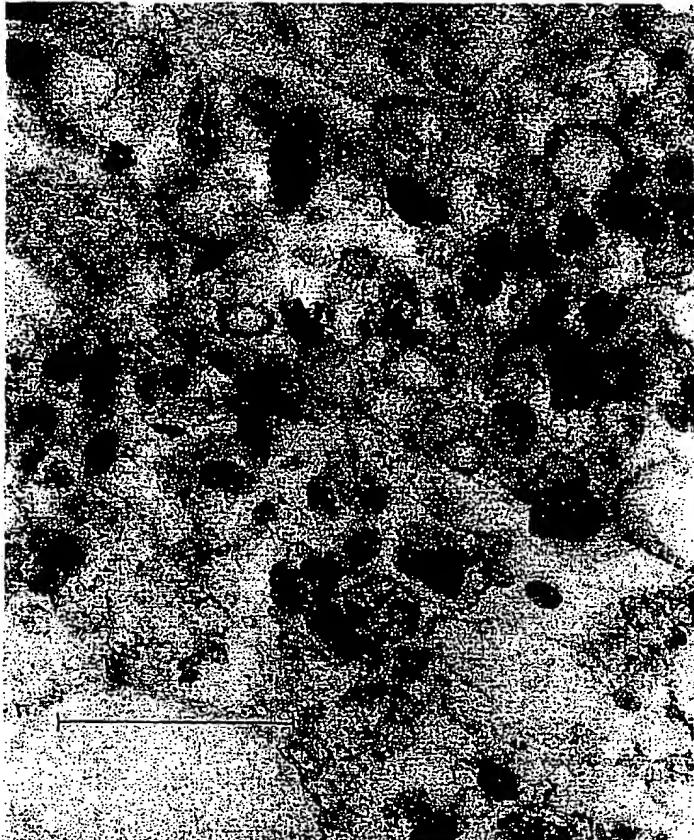
FIGURE 9.   The large-granule preparation of B-16 mouse melanoma (see FIGURE 8) in more detail, showing the various elements and particularly the different stages of melanization of melanosomes.   X80,000.

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and smooth membranes (FIGURE 15).   Typical electronmicrographs (FIGURES 16, 17, and 18) show that Fraction 2 consists mainly of small, dense particles that form large clusters and are not sharply outlined.   These particles are quite similar in both size and density to the 100 to 125 Å RNP (ribonucleoprotein) particles prepared from the microsomes of guinea pig liver.[23]   A few

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Various Fractions

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: 100 to 125 Å RNP (ribonucleopro-
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smooth membranes are also present. Fraction 3 consists mainly of smooth membranes (FIGURE 19). In Fraction 5, which consists in large part of melano-somes in the early stages of development, the internal structure of these particles and some smooth vesicles can be seen very clearly. Groups of striations that
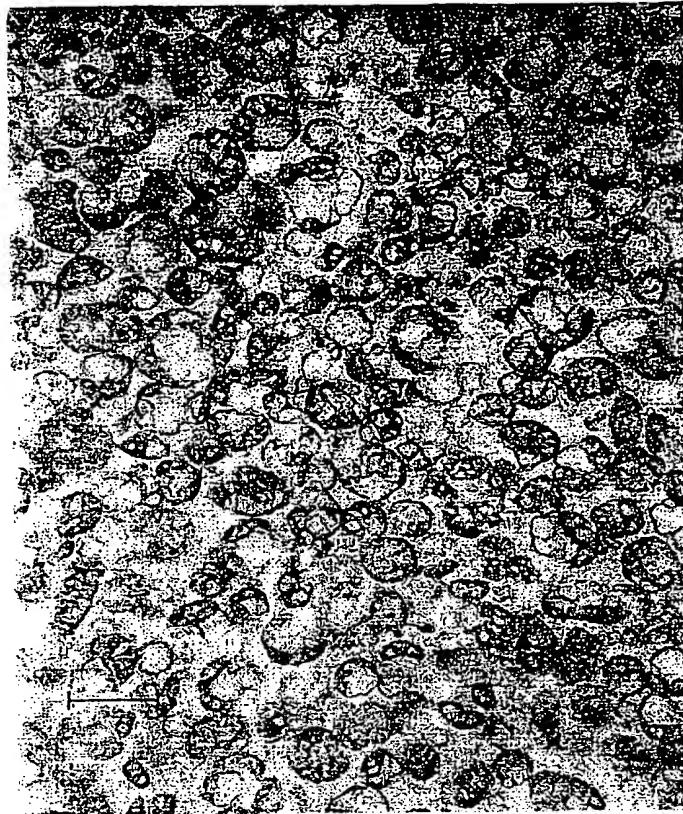
FIGURE 10. Electronmicrograph of Fraction 3, isolated from large-granule preparations of B-16 mouse melanoma by density-gradient centrifugation. Particles can be seen to be al-most exclusively mitochondria. ×32,000.

run parallel to the long axis of the granule (FIGURES 20 and 21) reveal the existence of a basic structure in which each striation has the appearance of a grid or lattice.

Electrophoretic Studies

In agar electrophoretic studies, the soluble tyrosinase isolated from the small- and the large-granule fractions was essentially the same and was found

to have two major components which could be clearly demonstrated by the acid blue-black stain for protein. The mobility of one of these active components was nearly the same as that of α-1-globulin of human serum, while the mobility of the other was the same as that of α-2-globulin (FIGURE 22). Both these major components showed a strong positive reaction with L-dopa.

The soluble tyrosinase also contained small amounts of protein which did not react with dopa, but were faintly stained by acid blue-black.

In Vivo Incorporation of Leucine-C¹⁴ by Soluble Tyrosinase Isolated from Mouse Melanoma at Different Intervals after Injection

The amount of L-leucine-C¹⁴ present in the soluble tyrosinase of each fraction 30, 45, 60, 90, 120, and 240 min. after injection of L-leucine-C¹⁴ is shown in FIGURE 23. The immediate, rapid labeling of the soluble tyrosinase isolated from the small-granule fraction is noteworthy. The specific activity of this soluble tyrosinase attained a maximum at 60 min. and decreased thereafter. The soluble tyrosinase isolated from the large-granule fraction, on the other hand, incorporated labeled amino acid slowly at first and reached its maximum specific activity 120 min. after injection. At 100 min., the specific activity of the soluble tyrosinase of the large-granule fraction rose above that of the small-granule fraction and remained above it thereafter.

Incorporation of Leucine-C¹⁴ into the Components Isolated from Soluble Tyrosinase by Agar Electrophoresis

Soluble tyrosinase was extracted from both the small- and the large-granule fractions isolated from mouse melanoma excised 1 hour after intraperitoneal injection of leucine-C¹⁴. FIGURE 24 shows the positions from which the samples were taken for counting and the position of the various compounds of human serum on the agar plates at the end of electrophoresis. Samples 1 to 5 were taken from areas in which both the protein and the dopa reactions were positive, where the protein reaction alone was positive, and where only plain agar was present. Tabulation of the results (TABLE 4) shows that there is definitive incorporation of leucine-C¹⁴ into the components in which both the protein and the dopa reaction are positive. Some incorporation also occurred in areas where the protein reaction was positive and the dopa reaction negative. Consequently, it appears that the incorporation of leucine-C¹⁴ by the various protein components of the soluble tyrosinase isolated by agar electrophoresis is quite uniform if the protein content of these components is used as a reference. The protein content of the soluble tyrosinase used in this experiment was approximately 1.5 μg. N in the small granules and 5.5 μg. N in the large granules.

DISCUSSION

The localization of mammalian tyrosinase activity in a suspension of cell particles has been reported by various investigators.[2,3] The results obtained when the distribution of tyrosinase activity in the different cell components was determined by means of recent separation methods are shown in TABLE 2. Using as a basis for calculation the experimentally obtained figure of 28 per cent recovery of tyrosinase activity in the nuclear fraction (TABLE 2), it appears

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l-globulin of human serum, while the
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that one might expect to recover from the purified preparation of nuclei 5.3
per cent of the total tyrosinase activity of whole tissue. Since the purified
preparation of nuclei has been estimated to be approximately 70 per cent pure,

FIGURE 11. Fraction 3 isolated from large-granule preparation of B-16 mouse melanoma
by density-gradient centrifugation (see FIGURE 10) in more detail. The arrangement of the
internal membranes can be seen. ×80,000.

the 5.3 per cent of tyrosinase activity recovered in the purified preparation
would have almost no significance. Actual experimental results indicate that
nuclei are of little importance in the localization of tyrosinase activity.

The experiments here described show that the so-called mitochondrial frac-
tion, the large-granule preparation of mouse melanoma obtained by the usual

method of cell fractionation, contains at least two structurally distinct elements, i.e., mitochondria and melanosomes. Density-gradient centrifugation made possible the separation of these particles into different zones according
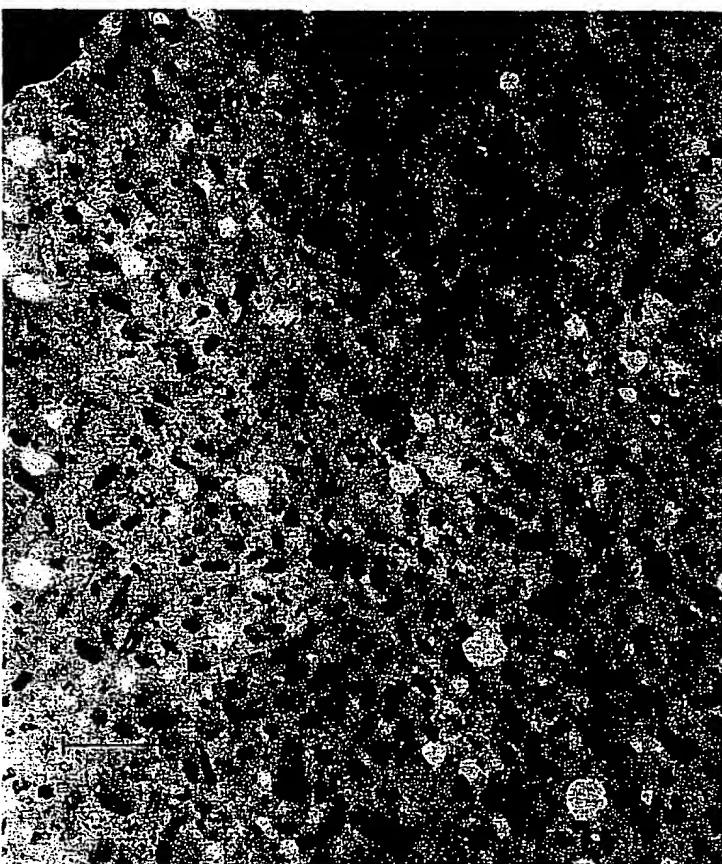

FIGURE 12. Electronmicrograph of Fraction 6 isolated from large-granule preparation of B-16 mouse melanoma by density-gradient centrifugation. This is a reasonably pure suspension of melanosomes. ×32,000.

to their respective densities. After centrifugation, the distribution of tyrosinase activity in the gradient tubes was entirely different from the distribution of the mitochondrial enzymes. Tyrosinase activity was found almost entirely in the lowest fractions in the tube. In electronmicrographs it can be seen that these lower fractions consist entirely of melanosomes. In preparations of B-16

DISTRIBUTION O
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Fraction	Protein	
	mg./ ml.	To m
Large granules	1.58	4.
1	0.02	0.
2	0.62	1.
3	0.15	0.
4	0.14	0.
5	0.57	1.

* At 103,000 g fr
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TABLE 3

DISTRIBUTION OF PROTEIN-NITROGEN, TYROSINASE, SUCCINOXIDASE AND GLUTAMATE
OXIDASE AFTER ULTRACENTRIFUGATION* OF LARGE-GRANULE PREPARATION OF B-16
MOUSE MELANOMA OVER DENSITY GRADIENT OF HYPERTONIC SUCROSE

Fraction	Protein nitrogen			Tyrosinase			Succinoxidase			Glutamate dehydrogenase		
	mg./ml.	Total mg.	Recovery per cent	Specific activity μl. O_2/hr./mg. N	Total μl. O_2/hr.	Recovery per cent	Specific activity μl. O_2/hr./mg. N	Total μl. O_2/hr.	Recovery per cent	Specific activity μl. O_2/hr./mg. N	Total μl. O_2/hr.	Recovery per cent
Large granules	1.58	4.75	100	35.1	167	100	769	3654	100	202	963	100
1	0.02	0.05	1.0	0	0	0	0	0	0	0	0	0
2	0.62	1.67	35.1	0	+†	0	1765	2943	80.6	640	1069	111
3	0.15	0.53	11.1	0	+†	0	0	0	0	92.4	49	5.1
4	0.14	0.53	11.1	301	160	95.8	64	34	0.9	26.4	14	1.4
5	0.57	1.7	35.7	141	240	143.7	21	36	0.98	37	63	6.5

* At 103,000 g for 1 hour.
† After incubation for 7 hours, the reaction mixture was slightly purple in color.
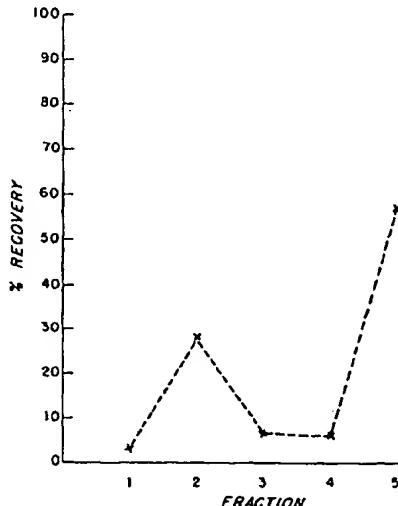
FIGURE 13. Tyrosinase activity of Fractions 1 through 5 separated from small-granule
preparation No. 1 of B-16 mouse melanoma by density-gradient centrifugation. Per cent
recovery is calculated in terms of the sum of the amounts recovered in all fractions. Each
point on the curve is the average of four observations. The various fractions are numbered
as in FIGURE 4B.

518   Annals New York Academy of Sciences

mouse melanoma, the sum of the tyrosinase activity of the various fractions was three times as great as the tyrosinase activity of the starting material, i.e., the large-granule preparation. This will be discussed elsewhere.

The bulk of the succinoxidase and glutamate oxidase activity was recovered in Fractions 2 and 3 respectively (the mitochondrial fractions). These are typical mitochondrial enzymes. No succinoxidase was found in Fractions 3,
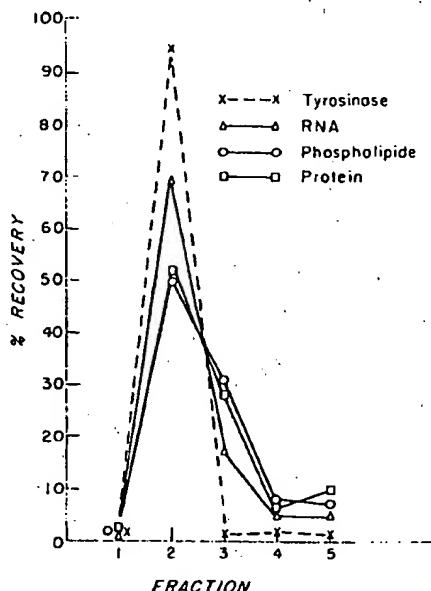

FIGURE 14. Tyrosinase activity and RNA, phospholipid and protein-nitrogen content of Fractions 1 through 5 separated from small-granule preparation No. 2 of B-16 mouse melanoma by density-gradient centrifugation. Per cent recovery is calculated in terms of the sum of the amounts recovered in all fractions. Each point on the curve is the average of four observations. The various fractions are numbered as in FIGURE 4B. Total recoveries were: protein-nitrogen, 94 per cent; tyrosinase activity, 125 per cent; RNA, 84.6 per cent; phospholipid, 90.8 per cent.

4, and 5 obtained by high density-gradient centrifugation of the large-granule preparation of B-16 mouse melanoma.

The experiments show that in suspensions of mouse melanoma there are granules which have the typical appearance of mitochondria under the electron microscope and contain a high concentration of succinoxidase and glutamate oxidase. These granules are found in a density layer in the gradient tube characteristic of all mitochondria studied, whatever their source. In addition, the tumor tissue contained more dense granules; these had the typical appearance of melanosomes and contained the greater part of the tyrosinase activity of the preparation. Some succinoxidase activity was found in this denser-

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A, phospholipid and protein-nitrogen content of
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granule fraction as the result of contamination with mitochondria, but by modified density-gradient centrifugation such contamination could be avoided and an essentially pure melanosome fraction obtained.

It is also possible, under the electron microscope, to recognize granules
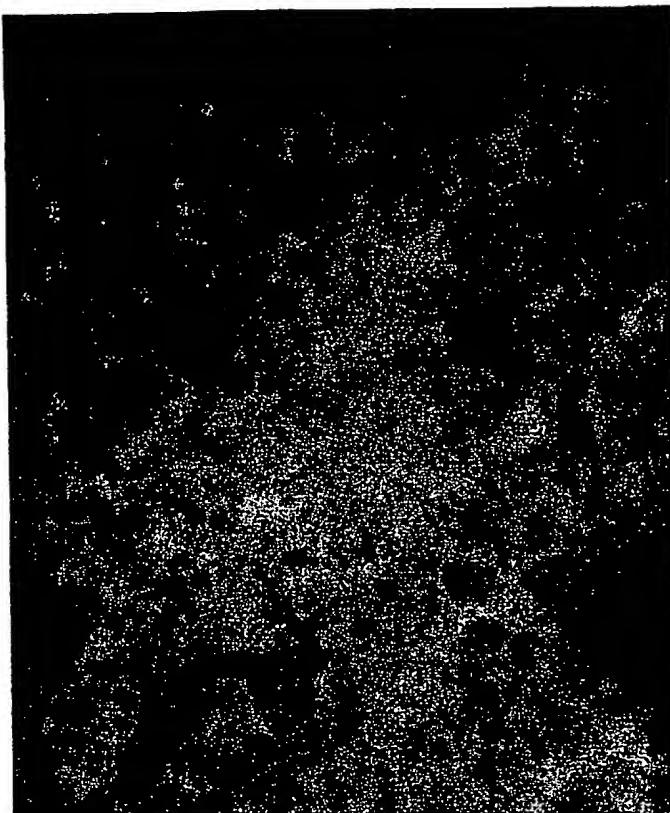
FIGURE 15. Electronmicrograph of small-granule preparation of B-16 mouse melanoma showing rough membranes, small electron-dense particles (ribonucleoprotein particles), smooth membranes, and a few melanosomes in the early stages of development. X32,000.

in process of melanization. FIGURES 8, 20, and 21 show internal structures present in melanosomes during their early stages of development. Groups of striations parallel to the long axis of the granule reveal the existence of a basic structure in which each striation has the appearance of a grid or lattice. The same internal structure can be seen in melanosomes from tissue preparations of Harding-Passey mouse melanoma and human skin (M. Seiji and J. B. Caul-

field, unpublished data, and Birbeck *et al.*[34]). Drochmans[19] reports that he has found similar striations with a basic crystallinclike lattice structure in the melanin granules of cat and human skin. Birbeck and Barnicot,[35] using human hair bulbs, and Dalton[5] and Wellings,[14,15] using mouse and human melanoma

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FIGURE 16. Electronmicrograph of Fraction 2 isolated from small-granule preparation of B-16 mouse melanoma by density-gradient centrifugation. Particles are almost exclusively small and dense. ×32,000.

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respectively, have pointed out that in their electronmicrographs there could be seen a series of steps in the formation of the melanin granule. They postulate that as the small vesicles of the Golgi zone increase in size, dense membranous structures become evident within the bounding membrane of the vesicles. These internal membranes appear as concentric, spiral, or parallel strands in their presumed cross sections. These gradually become considerably

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thicker and their density increases until it is approximately the same as that of
the outer membrane of the vesicle in its early stage. Later the spaces between
the inner membranes are filled in, so that eventually the granule becomes
uniformly dense and structureless. Although the dense material seen in
electronmicrographs may not be precisely the same as melanin, it seems quite

2 isolated from small-granule preparation
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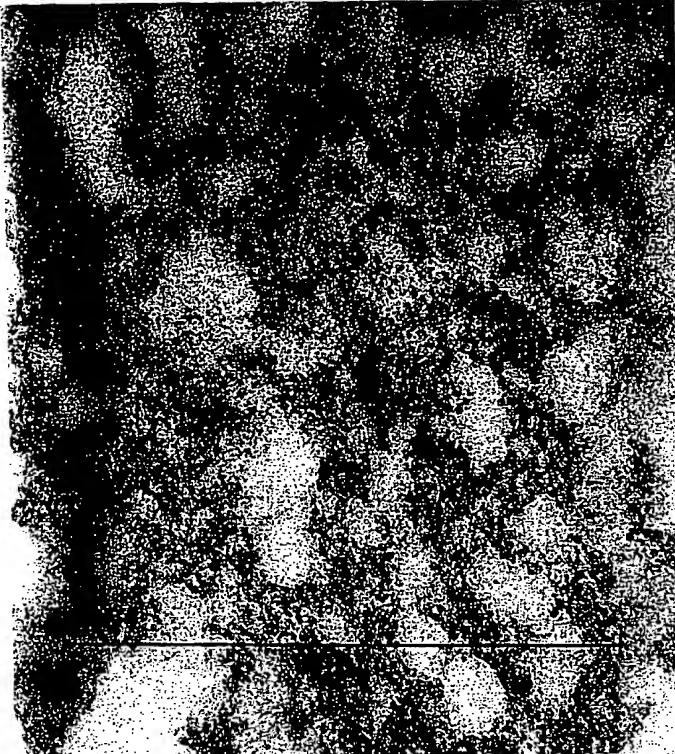
FIGURE 17. Electronmicrograph of Fraction 2 isolated from the small-granule preparation
of B-16 mouse melanoma by density-gradient centrifugation. The particles are quite similar in size and density and show some clumping. ×160,000.

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safe to consider that the changes seen may be various stages in the gradual
transformation of the initial vesicles into structureless, completely melanized
melanin granules.

Moyer,[6] on the basis of his electron-microscope studies of the retinal pigment
of the mouse embryo, has speculated, on the other hand, that melanin granules
may form in intracisternal dilations of the endoplasmic reticulum and that
subsequently there appear "hollow ellipsoids" that are bounded by a typical

double membrane and contain small vesicular and fibrous elements. He thinks that the ellipsoids thus produced become filled with branching fibers that lie parallel to their long axis, and that melanin polymerizes on these fibers

his intracisternal communication).

Although the
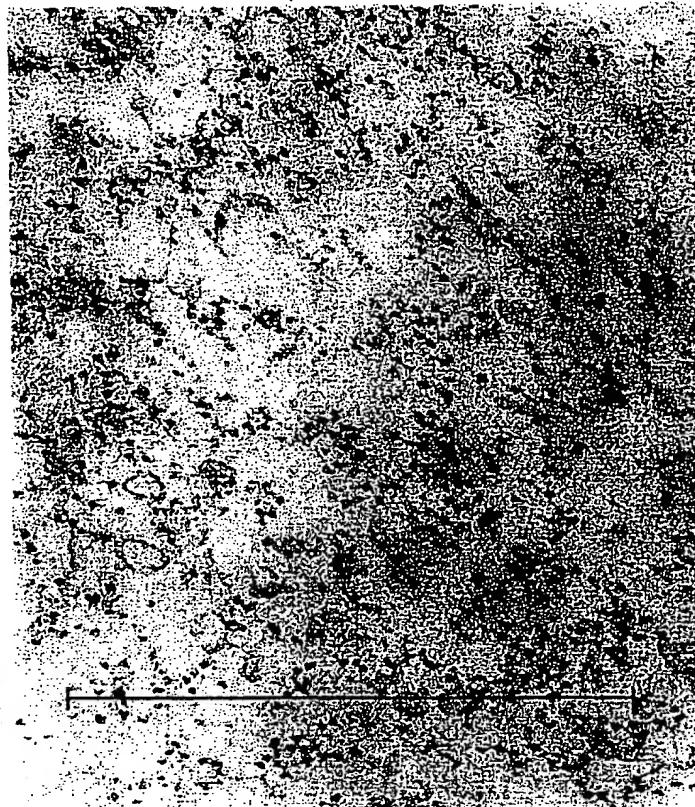
FIGURE 18. Electronmicrograph of Fraction 2 isolated from the small-granule preparation of B-16 mouse melanoma by density-gradient centrifugation. The field was selected to show the 100–150 Å, electron-dense ribonucleoproteinlike particles and some smooth membranes. ×160,000.

FIGURE 19. Ele B-16 mouse melano sist mainly of smoot

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until the underlying structure is completely obscured and the granule has reached morphological maturity. Moyer has found no evidence to support the older theories of the mitochondrial, nuclear, or Golgi origin of melanin granules. Recently, however, he has noted the existence of a close relationship between ribonucleoprotein particles and the fibrous structure observable in the early stages of melanin-granule formation. This may lead him to change

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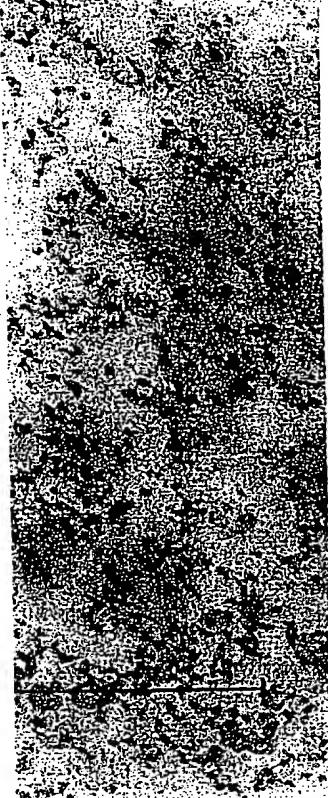
his intracisternal theory of the origin of melanin granules (F. Moyer, personal communication).

Although the origin of melanosomes is still unknown, evidence has accumu-

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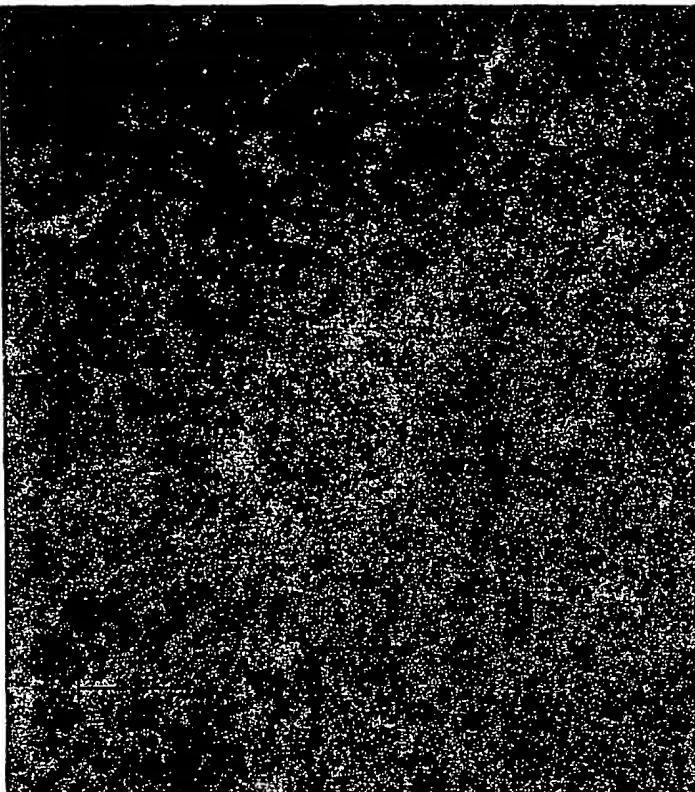
FIGURE 19. Electronmicrograph of Fraction 3 isolated from small-granule preparation of B-16 mouse melanoma by density-gradient centrifugation. This fraction was found to consist mainly of smooth membranes.

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lated that supports the concept that melanization of melanosomes within the melanocyte is a stepwise process.

The regular presence of tyrosinase activity in the small-granule fraction raises the following questions. Is the small-granule fraction contaminated with small melanosomes? Do the cell components (*e.g.*, ribosomes, endoplasmic reticulum) present in the small-granule fraction really contain tyrosinase activity? To answer these questions, the particles present in the small-granule fraction,

concluded that Fraction 2 derived from the small-granule preparation is not contaminated by melanosomes and that the particles in this fraction and melanosomes are disparate in nature. The difference between the chemical composition of melanosomes and that of Fraction 2 is also quite definite (M. Seiji and T. B. Fitzpatrick, unpublished data).

The microsome fraction isolated from various mammalian tissues contains small, electron-dense particles.[31,36,37] The appearance of particles of this type
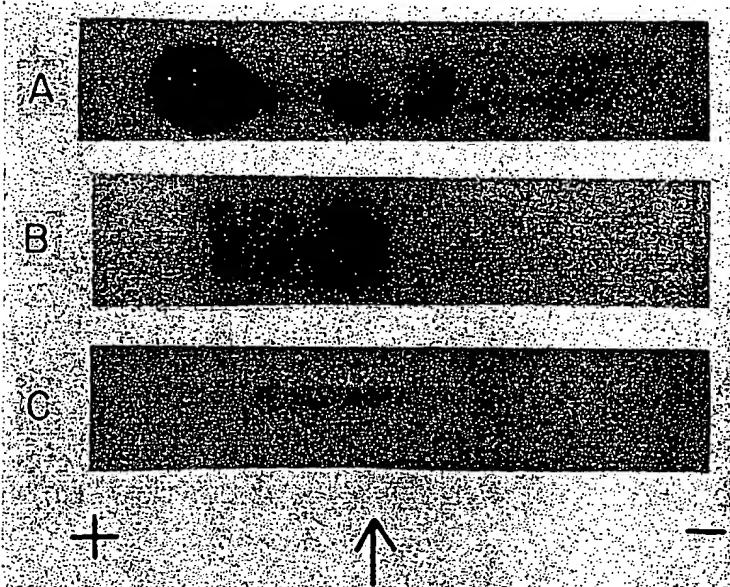
FIGURE 22. Agar electrophoretic pattern of soluble tyrosinase isolated from B-16 mouse melanoma. A = human serum, B = soluble tyrosinase stained by acid blue-black, C = dopa reaction positive. Electrophoresis took place at 4° C. with veronal sodium-HCl buffer at pH 8.6 at ionic strength 0.05. The patterns shown were obtained during a 4-hour run at 8 mAmp. Arrow indicates the starting position. Two major components in which both the dopa and the protein reaction were positive can be seen at B and C.

in the Fraction 2 isolated from B-16 mouse melanoma was very similar to that of particles isolated from the liver and pancreas of guinea pigs.[23] Small ribonucleoprotein particles (RNP-particles) separated biochemically from the microsome fraction with deoxycholic acid contain a high concentration of ribonucleic acid.[36,38] The RNA content of RNP-particles isolated from B-16 mouse melanoma was found to be 40 per cent. Chemical analysis revealed that Fraction 2 contained 13 to 20 per cent ribonucleic acid in terms of dry weight, and some phospholipid. Although Fraction 2 in these experiments may not have been a pure suspension of RNP-particles, it did contain small

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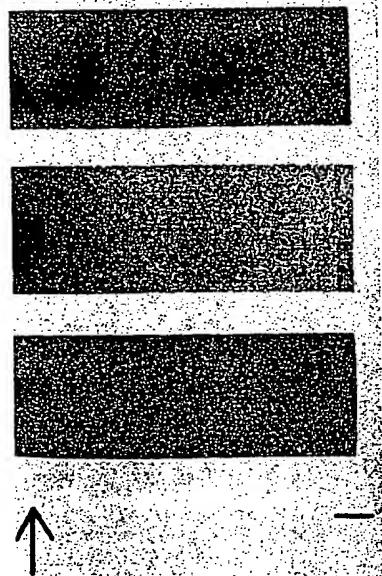
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particles that look like RNP-particles and it had a high content of ribonucleic
acid. It is possible, therefore, to consider that its granules are similar in
nature to RNP-particles.

The use of radioactive tracers for the intensive investigation of protein
biosynthesis during the last decade has been based on the premise that micro-
somes, more specifically RNP-particles, are the intracellular site of protein
synthesis. The essential role of RNA in protein synthesis has been well demon-
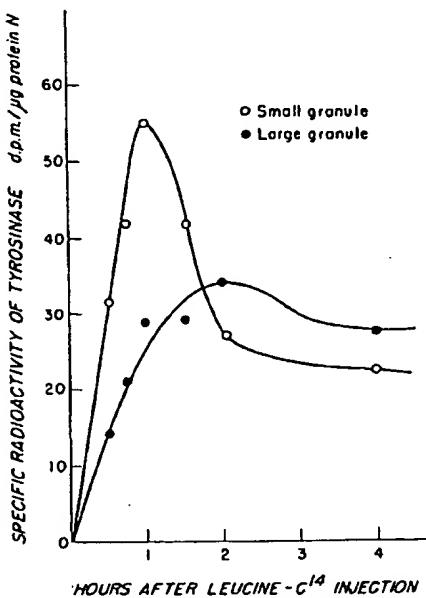
FIGURE 23. Comparison of the rate of incorporation of leucine-C[14] into the soluble ty-
rosinase isolated from the small-granule preparation and the large-granule preparation of
B-16 mouse melanoma. The specific radioactivity (disintegration per minute per μg. pro-
tein-nitrogen) was determined at various intervals after injection of leucine-C[14] into living
mice with melanoma.

strated.[30] It is therefore necessary to consider in terms of protein synthesis
the significance of tyrosinase activity in Fraction 2 of the experiments described;
in other words, are the particles found in Fraction 2 the site of tyrosinase bio-
synthesis?

If melanosomes are accepted as the site of melanogenesis in the melanocyte,[17]
is tyrosinase, the enzyme responsible for the formation of melanin from tyrosine,
synthesized in the melanosome? Is tyrosinase derived from its precursor in
the melanosome by a mechanism similar to that involved in the formation of
α-chymotrypsinogen in the pancreas?

Incorporation of radioactive amino acids in vivo has been repeatedly used to

demonstrate the synthesis of specific proteins such as ribonuclease,[39,40] trypsinogen, and α-chymotrypsinogen.[39,41,42] Isolation of specific proteins from various cell fractions after *in vivo* or *in vitro* labeling has also been used in several studies as a means of locating the intracellular sites of protein synthesis.[42,43,44]
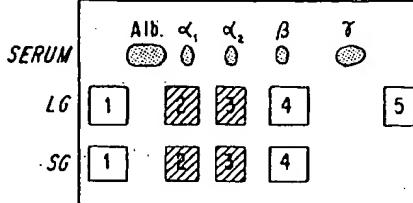

FIGURE 24. Radioactivity of electrophoretically separated components of soluble tyrosinase prepared from the large and small granules isolated from B-16 mouse melanoma. Large granules and small granules were obtained from B-16 mouse melanoma which was excised 1 hour after leucine-C[14] had been injected into the peritoneal cavity. Electrophoresis took place at 4° C. with veronal sodium-HCl buffer at pH 8.6 and ionic strength 0.05. The patterns shown were obtained during a 4-hour run at 8 mAmp. Human serum was used to provide a reference of electrophoretic mobility. Diagram shows the areas from which samples were taken from the agar plate for the counting of radioactivity. 1 = area where reaction to acid blue-black stain for protein was weakly positive and reaction to L-dopa was negative; 2 and 3 = areas where both protein and L-dopa reactions were strongly positive; 4 = area where protein reaction was positive and L-dopa reaction negative; 5 = area without protein (plain agar); *LG* = large granules; *SG* = small granules.

TABLE 4

RADIOACTIVITY OF ELECTROPHORETICALLY SEPARATED COMPONENTS (1 TO 4)* OF SOLUBLE TYROSINASE PREPARED FROM THE LARGE AND SMALL GRANULES ISOLATED FROM B-16 MOUSE MELANOMA

In This Experiment, the Protein Content of Soluble Tyrosinase Obtained from the Small Granules was Approximately 1.5 μg. Protein-Nitrogen; from the Large Granules, 5.5 μg. Protein-Nitrogen

Source of soluble tyrosinase	Radioactivity (cpm)				
	1	2	3	4	5
Large granules	2.5	19.1	18.1	9.9	1.1
Small granules	5.7	16.7	7.9	2.5	

* See FIGURE 25.

The *in vivo* incorporation of leucine-C[14] into the soluble tyrosinase of various cell particles has been determined. Because the method of isolating soluble tyrosinase from cell particles is inefficient, the large-granule fraction and the small-granule fraction, respectively, were used as starting material for the isolation process. This made it possible to obtain sufficient soluble tyrosinase for experimental purposes. The curve of incorporation into tyrosinase from the small-granule fraction rose more rapidly at first than the curve of incorporation into tyrosinase from the large-granule fraction, reaching its peak 60 minutes

proteins such as ribonuclease,[39,40] trypsino-
Isolation of specific proteins from various
-o labeling has also been used in several
tracellular sites of protein synthesis.[42,43,44]

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ABLE 4

Separated Components (1 to 4)* of Soluble
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Melanoma

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atein-Nitrogen; from the Large Granules, 5.5
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	Radioactivity (cpm)		
2	**3**	**4**	**5**
9.1	18.1	9.9	1.1
6.7	7.9	2.5	

-C[14] into the soluble tyrosinase of various
Because the method of isolating soluble
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after injection of the leucine-C[14], and then fell below the curve of incorporation
into tyrosinase from the large-granule fraction (FIGURE 23). According to
Reiner's mathematical analysis,[45] the pattern formed by these two curves is
quite suggestive evidence of a precursor-product relationship between the small
granules and the large granules in terms of tyrosinase activity.

In electrophoretic studies, the soluble tyrosinase isolated from these two
fractions was essentially the same: it contained two major components, both
of which contained tyrosinase activity and both of which incorporated leu-
cine-C[14] (FIGURE 24). Therefore, although the soluble tyrosinase studied in
this series of experiments was not pure, findings make it reasonable to suppose
that leucine-C[14] was incorporated into the enzyme.

Under the electron microscope, the appearance of melanosomes is quite
different from that of the small particles in Fraction 2, and no morphological
evidence has been found in the melanin-forming cell of transitional forms be-
tween the small particles and melanosomes. The chemical composition of
melanosomes differs from that of Fraction 2: the RNA content of Fraction 2
was as much as 24 times greater than that of melanosomes which contain only
4 µg. RNA-P/mg. protein-nitrogen. At the present time, therefore, it seems
very unlikely that tyrosinase is synthesized within the melanosome.

It has been suggested that substances formed in the secretory cell may be
synthesized in or on RNP-particles that are attached to the membrane of the
endoplasmic reticulum, and then transfered across the limiting membrane of
the network and deposited in its interstices. In the form of granules or in
solution, the product of secretion might move through the channels of the
reticulum to the Golgi zone to be separated there into quanta, each surrounded
by its own membranous envelope within which condensation would take place;
in other words, each quantum might become a secretory granule.[42,46,n]

At the present time, although a few mechanisms involved in the formation
of the melanosome remain unknown (*e.g.*, condensation of tyrosinase in the
region of the centrosphere and formation of the network), it can be assumed
that in the melanocyte tyrosinase is synthesized in the ribonucleoprotein
particles and transferred through the endoplasmic reticulum or vesicles to the
Golgi area where it is quantized. Each quantum might then acquire a mem-
branous envelope within which the tyrosinase is condensed and loaded onto or
into the network. Structures of this sort, *i.e.*, melanosomes, gradually become
melanized within the cytoplasm by melanin, the product of the tyrosine-
tyrosinase reaction. As melanin accumulates on the internal network, the
external membrane gradually becomes thicker and the density of the enclosed
particles increases until the interstices of the inner network have been filled in.
Eventually, each granule becomes a uniformly dense and structureless unit,
the melanin granule, which is incapable of further melanin formation (FIGURE
25).

For several decades, investigations of melanin formation have been unre-
warding because knowledge of the basic dynamic mechanism of melanogenesis
in the melanocyte was lacking. Now it is possible to describe the actual process
of melanin biosynthesis. The melanosome concept of melanogenesis, evolved
from the experimental data reported here, provides a comprehensive view of
mammalian melanogenesis: tyrosinase, the enzyme responsible for melanin

formation, is synthesized on the RNP-particles and loaded into melanosomes, where melanization takes place, with ensuing gradual transformation of the melanosomes into large amorphous structures, the melanin granules. It seems that in any study of the factors that regulate melanogenesis, e.g., the hormones or in any explanation of differences in the rate and amount of melanin forma-
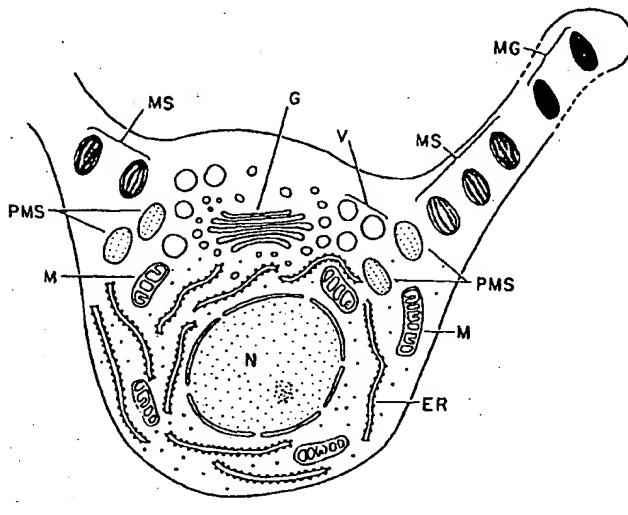
MELANOCYTE

FIGURE 25. Diagram of a melanocyte, showing melanin granules in various stages of development. The polypeptides that eventually become "tyrosinase" are synthesized in small granules (presumably ribonucleoprotein particles), transferred, perhaps along the endoplasmic reticulum (ER), to the Golgi area (G), and condensed into "pro-tyrosinase," the secondary and tertiary structures of which tyrosinase is composed. In the Golgi area, pro-tyrosinase is separated into small units, each of which becomes surrounded by a membranous envelope (V). Within each envelope, the pro-tyrosinase molecules become aligned in an ordered pattern. When this has occurred, the unit is known as a "premelanosome" (PMS). The pro-tyrosinase then becomes active, i.e., becomes tyrosinase, melanin biosynthesis begins and the particle is known as a "melanosome" (MS). As melanin gradually accumulates in the cytoplasm, the melanosome is eventually transformed into a uniformly dense and structure-less particle, the "melanin granule" (MG) in which no tyrosinase activity can be detected (FIGURE 1).

tion in normal and neoplastic cells one must consider: (1) the rate of tyrosinase synthesis on the RNP-particles; (2) the rate of melanosome production; and (3) the various factors that regulate the biosynthesis of melanin from tyrosine in the melanosome.

SUMMARY

The various components of B-16 mouse melanoma have been isolated by differential centrifugation, tested individually for tyrosinase activity and subjected to biochemical assay and examination under the electron microscope.

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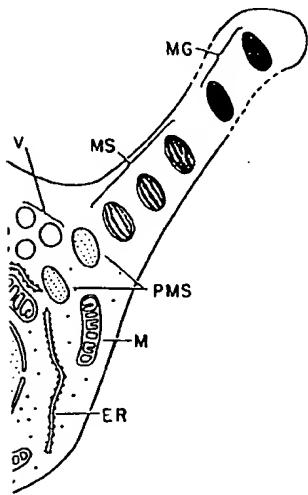
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Twenty-eight per cent of the total tyrosinase activity of the initial homogenate of melanoma tissue was recovered in the nuclear fraction. Calculations show that if the nuclear fraction has been purified, it should theoretically be possible to recover 5.3 per cent of the total tyrosinase activity of whole melanoma tissue from the nuclear fraction. Since the purified preparation of nuclei studied contains approximately 70 per cent nuclei, the amount of tyrosinase activity actually recovered in the purified preparation is thought to be almost without significance.

The large-granule preparation has been separated by density-gradient centrifugation into several fractions. The granules which have the typical appearance of mitochondria under the electron microscope contain a high concentration of succinoxidase and glutamate oxidase, both of which are typical mitochondrial enzymes. These granules are found in the gradient tube in a density layer where mitochondria are characteristically found, whatever their biological source. The granules which, under the electron microscope, had the typical appearance of melanosomes have been found to contain the bulk of the tyrosinase activity of the large-granule preparation. It is concluded that melanosomes and mitochondria are distinct cytoplasmic constituents of the melanin-forming cell and that each of these two constituents is equipped with its own characteristic enzymes.

Although a large part of the tyrosinase activity present in the original tissue homogenate was found in the large-granule preparation, a certain amount of tyrosinase activity was also regularly present in the small-granule preparation.

One type of particle isolated from the small-granule preparation by the density-gradient method resembled RNP-particles under the electron microscope; it also contained a high concentration of RNA and phospholipid as well as tyrosinase activity.

The rate of *in vivo* incorporation of leucine-C^{14} into soluble tyrosinase isolated from both the small-granule fraction and the large-granule fraction has also been determined. The pattern of incorporation of leucine-C^{14} by the soluble tyrosinase of these two fractions is adequate proof of their precursor-end-product relationship.

The data obtained in this study of the morphology and biochemistry of cell components and the *in vivo* incorporation of leucine-C^{14} by soluble tyrosinase are all compatible with the hypothesis that tyrosinase is synthesized in small granules, which are presumably RNP-particles, and subsequently transferred through the endoplasmic reticulum to melanosomes where it is stored. Melanosomes thus formed are gradually melanized until they eventually become uniformly dense and structureless particles, the melanin granules, which are incapable of further melanin formation, and eventually are excreted into other cells.

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Table 1 Effect of hGHRF(1-29)NH₂ on anterior pituitary stores of GH

	(n)	Wet weight (mg)	Anterior pituitary GH content (µg)	GH concentration (µg per mg wet wt)
a Normal females				
Saline	(8)	9.8 ± 0.3	613 ± 36	62 ± 3
Continuous GHRF	(8)	10.2 ± 0.7	623 ± 68	61 ± 5
Pulsatile GHRF	(8)	10.3 ± 0.4	834 ± 75*	81 ± 7*
b MSG-treated males				
Saline	(8)	4.5 ± 0.5	522 ± 119	104 ± 17
Continuous GHRF	(8)	4.6 ± 0.5	577 ± 112	115 ± 14
Pulsatile GHRF	(8)	5.3 ± 0.6	680 ± 161	115 ± 20

Anterior pituitary glands were removed, weighed, homogenized individually in 1 ml phosphate-buffered saline pH 7.4, centrifuged for 10 min at 13,000g and the supernatants assayed for rat GH by radioimmunoassay (RIA), using six doubling dilutions in duplicate. See text for an explanation of the different treatment groups. In female rats (*a*), pulsatile hGHRF(1-29)NH₂ for 12 days increased pituitary GH content and concentration. In male MSG-treated rats (*b*) hGHRF(1-29)NH₂ had no significant effect on pituitary GH.
* $P < 0.05$ vs. both saline and continuous GHRF.

of uniformity and availability, together with an intact hypothalamo-hypophysial system. The possibility that prolonged pulsatile GHRF treatment might alter reproductive cycles in the female rat, and thus indirectly influence body growth must be considered, although this explanation is unlikely to account for the stimulation of growth produced in male rats by pulsatile GHRF. The simplest explanation for our results is that effective GHRF levels in the hypophysial portal vessels are achieved only by giving the peptide in pulses. Continuous infusions of GHRF require higher total dose rates to stimulate GH release and can result in rapid depletion of pituitary GH stores[20]. In the case of MSG-treated animals, we are replacing the depleted GHRF secretion, whereas in normal female rats we are presumably overriding endogenous GHRF secretion. Whether or not the endogenous secretory profile of GHRF in male rats shows 3-hourly episodes, an exogenous GHRF peptide given in 3-hourly pulses produces a plasma GH profile which mimics the normal male pattern. We have previously shown in hypophysectomized male rats that the growth responses to a given dose of GH are greater if the GH is given in 3-hourly pulses[12]. The pattern of GH secretion is less pulsatile in female rats, and this may partly underlie their slower growth rates[21]. We reasoned that maintaining a male pattern of GH secretion might increase the growth rate of normal females towards that of the male rat. This proved to be the case.

Both normal female rats and MSG-treated male rats may provide useful data when considering the treatment of GHRF-deficient children with pulsatile GHRF[7], especially where this has to be given by the subcutaneous route, which inevitably reduces the pulsatility. Our demonstration that a more effective GH secretory profile can be imposed in normal, intact (female) animals by patterned exogenous GHRF may well have applica-

bility in animal husbandry, provided suitable simple means for achieving the optimal pattern of administration can be found. The obvious corollary of this result is that a sub-optimal infusion regime might impose a less effective growth rate, which could conceivably be useful therapeutically in children with excessive rates of growth. At its simplest level, the chronically cannulated female rat offers a convenient model in which to test the effects of different routes and patterns of administration of GHRF, its analogues and inhibitors on whole body growth.

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Table 2 Effect of hGHRF(1-29)NH₂ on tail and bone growth in MSG-treated rats

	(n)	Tail growth (mm)	Proximal tibial growth (µm per day)
Saline	(8)	15.4 ± 1.3	137 ± 12
Continuous	(8)	18.2 ± 2.6	137 ± 8
Pulsatile	(8)	20.6 ± 1.3*	159 ± 7†

Tail lengths of MSG-treated rats were measured[22] under anaesthesia at the beginning and end of the infusion experiment (see text). The differences between these measurements are shown as mean ± s.e.m. Immediately before the infusions began, each rat received an injection of oxytetracycline (2 mg per 100 g body weight, i.v.). At the end of the experiment, the proximal tibias were dissected out and cumulative bone growth assessed by fluorescence microscopy according to the method of Hansson *et al.*[13]. Results were analysed using paired *t*-tests against littermate controls.
* $P < 0.001$ vs. saline; † $P < 0.05$ vs. continuous GHRF.

Tissue-specific expression of rat myosin light-chain 2 gene in transgenic mice

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One approach to determining how the differential expression of specific genes is regulated in higher organisms is to introduce cloned copies of the genes (or parts of the genes) into the genomes of individual organisms from the very beginning of their development. The way in which the exogenous genetic information behaves during the development of the experimental organisms can then provide a means of defining the DNA sequences that restrict the

expression of the gene to specific cell types and times of development. So far, several different genes have been introduced into the genomes of mice[1-15], but in only a few cases have the exogenous genes retained the tissue specificity of expression of the equivalent endogenous genes[12,14,15]. I report here that in two out of three 'transgenic' mice carrying copies of the rat gene for skeletal muscle myosin light chain 2, the exogenous gene is expressed specifically in skeletal muscle cells. The sequences contained in the cloned copy of the myosin light-chain 2 gene used in these experiments are thus sufficient to confer a tissue-specific pattern of expression.

A recombinant plasmid (pRH3MLC)[16] containing the rat skeletal muscle myosin light-chain 2 (MLC2) DNA sequences in their entirety, in a 4.7-kilobase (kb) DNA fragment, was cut with HindIII to release the rat DNA sequences from the plasmid DNA and used for injection into fertilized mouse eggs (Fig. 1a). Superovulated females of (BALB/c × C57BL/6J) F₁ were mated to males of (C57BL/6J × DBA) F₁. On the day after mating, fertilized eggs were recovered from the oviducts. The male pronuclei were microinjected with 1-2 pl containing ~500 molecules of the plasmid DNA. Eggs that survived the microinjection (~50%) were implanted into the oviducts of pseudopregnant CD-1 females for further development. Spleen DNA samples from 26 neonatal mice were analysed by Southern blot hybridization and four mice containing pRH3MLC sequences were identified. DNA isolated from spleens of these mice (designated MLC₁, MLC₂, MLC₃, MLC₄) and a control mouse was digested with HindIII, EcoRI or XbaI. Figure 1b shows an autoradiogram of a Southern blot of the fractionated DNA, hybridized to ³²P-labelled pRH3MLC DNA. In addition to the expected 4.7- and 4.3-kb DNA fragments in the HindIII digest, several minor fragments that hybridized to the probe could be

observed in DNA from mice MLC₁, MLC₂ and MLC₄, these fragments resulting from either junction fragments with cellular DNA or rearranged sequences.

As the pRH3MLC DNA was cut with HindIII before microinjection, multiple orientations of MLC2 and pBR322 DNA sequences were expected (Fig. 1). For example, in the EcoRI digests, the 1.6-kb DNA fragment seen in the four transgenic mice is a product of cleavage at the internal EcoRI sites of the MLC2 gene. The 2.0-kb DNA fragment in transgenic mice MLC₁, MLC₃ and MLC₄ is a product of cleavage at the 3' end EcoRI site of MLC2 gene, when it is integrated next to pBR322 DNA in a head-to-tail configuration. This fragment is undetectable in mouse MLC₂ DNA; instead a predominant 6.3-kb DNA fragment appears, indicating that most of the MLC2 DNA molecules in this mouse are arranged in tandem arrays with pBR322 DNA in a head-to-head orientation. Similarly, in the XbaI digests, the 3.9-kb DNA fragment is a product of cleavage at the two sites when pBR322 DNA is flanked on both sides with MLC2 DNA sequences. Analysis of the restriction pattern indicated that most of the copies of the injected DNA are integrated tandemly in the mouse genome. The number of pRH3MLC DNA copies in each mouse was estimated by comparing the intensity of the hybridized fragments with those of known amounts of plasmid DNA. Thus, mice MLC₁, MLC₂, MLC₃ and MLC₄ contain ~20, 10, <1 and 16 copies per cell, respectively.

To examine the transmission of the inserted DNA sequences to progeny, each of the four transgenic mice was mated with a normal mouse. Southern blot hybridization to spleen DNA from the progeny of MLC₁, MLC₂ and MLC₄ revealed that the inserted DNA sequences were transmitted faithfully through the germ line to ~50% of the progeny (data not shown; see Fig. 2), the expected frequency if the majority of the multiple MLC2 copies are integrated tandemly at a single site of one chromosome.

In contrast to the mendelian inheritance of pRH3MLC DNA sequences in mice MLC₁, MLC₂ and MLC₄, mouse MLC₃ did not transmit the injected DNA to any of its first 60 offspring. This female mouse contains <1 copy per cell of the inserted DNA (Fig. 1b) and is apparently a mosaic in which the injected DNA is present in only a small proportion of its somatic cells.

To investigate whether the expression of the rat MLC2 gene in transgenic mice was tissue-specific, RNA was extracted from skeletal muscle and other organs from the progeny of two transgenic mice. The presence of rat MLC2 transcripts in these RNA preparations was determined by the S₁ endonuclease technique, using as a probe the coding strand of the 630-base pair (bp) HinfI/HinfI DNA fragment derived from the 5' region of the rat MLC2 gene and labelled at the 3' end with reverse transcriptase (see Fig. 3). In stringent hybridization conditions
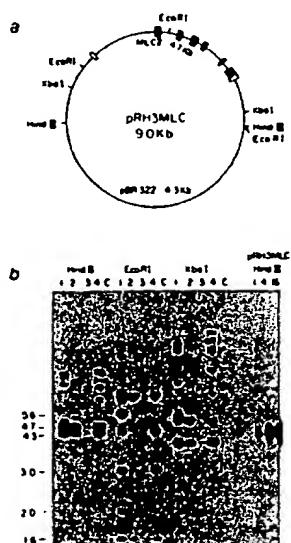

Fig. 1 Map of plasmid DNA pRH3MLC and Southern blot analysis of DNA from four transgenic mice. a, The 4.7-kb HindIII DNA fragment containing the rat MLC2 gene was cloned into the HindIII site of pBR322 (ref. 16). Boxes indicate the positions of the seven exons; empty boxes represent the 5'- and 3'-untranslated regions; solid boxes represent coding sequences. b, Total spleen DNA (10 μg) from the four transgenic mice (1-4) and a control mouse DNA (C) was digested with HindIII, EcoRI or XbaI. The DNA fragments were fractionated on a 0.8% agarose gel, transferred to nitrocellulose filter and hybridizes with ³²P-labelled pRH3MLC DNA. The blot was washed in stringent conditions (0.1 × SSC, 0.2% SDS at 75 °C for 2 h) not allowing detection of the mouse gene. Plasmid pRH3MLC DNA samples digested with HindIII corresponding to 1, 4 and 16 copies per diploid mouse genome were included to estimate the copy number of inserted DNA in transgenic mice. Molecular sizes are shown in kb on the left.
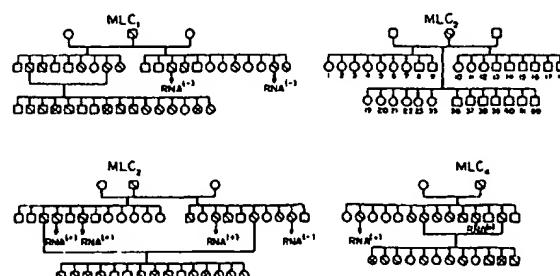

Fig. 2 Inheritance of pRH3MLC sequences in the four transgenic mice. Each transgenic mouse was mated with normal mouse. Heterozygous male and female progeny of MLC₁ and MLC₂ mice were mated to give rise to normal homozygous mice. Mouse MLC₃ is probably a mosaic. Squares represent males, circles females, intersecting diagonals homozygous mice, single diagonals heterozygous mice. Mice denoted RNA⁽⁺⁾ contained MLC2 transcripts in skeletal muscle whereas mice denoted RNA⁽⁻⁾ did not.
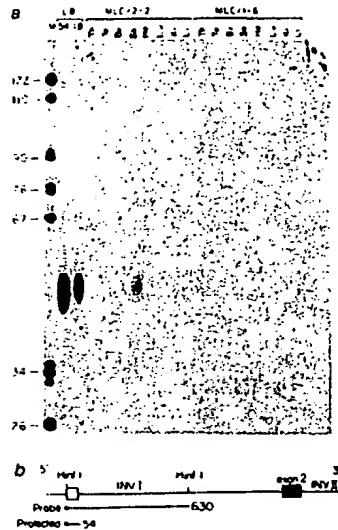
Fig. 3 S₁ analysis of rat MLC2 RNA in the progeny of transgenic mice MLC₁ and MLC₂. *a*, 25 μg total RNA from the indicated tissues and total RNA from differentiated cultures of the myogenic cell line L8 were hybridizied at 46 °C for 16 h to a 3'-end-labelled *Hin*fl/*Hin*fl DNA fragment prepared from pRH3MLC DNA (see scheme in *b*). Th, thymus; Te, testes; Br, brain; Sk, skeletal muscle; He, cardiac muscle; Lu, lung; Ki, kidney; Li, liver. Hybridization reactions were incubated with 5,000 U S₁ nuclease (Miles) for 30 min at 42 °C. S₁-resistant hybrids were sized on 5% acrylamide/7M urea gel. *b*, Map of the 5' region of the rat MLC2 gene and the hybridization probe. Boxes, exons; solid lines, introns and 5'-flanking region; open box, 5'-untranslated exon; solid box, first coding exon. The 3' label is indicated by an asterisk and the lengths of the probe and the protected fragment are also indicated.

Fig. 4 The rat MLC2 gene is initiatied correctly in progeny of transgenic mice MLC₂ and MLC₄. Total RNA from skeletal muscle of male (♂) and female (♀) progeny of transgenic mice MLC₁, MLC₂ and MLC₄. RNA from skeletal muscle of control mouse and from differentiated cultures of the myogenic cell line L8 were hybridized with 5'-end-labelled *Sau*3A/*Eco*R1 DNA fragment (see *c*). RNA (μg) in each hybridization reaction is indicated. *a*, Hybridization performed at 42 °C and S₁ digestion at 37 °C; *b*, hybridization at 37 °C and S₁ digestion at 32 °C. After treatment with S₁ nuclease, the resistant hybrids were sized on 5% acrylamide/7 M urea gels. *c*, Map of the 5' region of the rat MLC2 gene and the probe used in the hybridization. Boxes, exons; solid line, introns and 5'-flanking region; open box, 5'-untranslated exon; solid box, first coding exon. The 5' label is indicated by an asterisk and the lengths of the probe and the protected fragment are shown.

(hybridization at 46 °C and S₁ digestion at 42 °C), cross-hybridization to the endogenous mouse MLC2 transcripts did not occur. Authentic rat transcripts protect a 54-nucleotide DNA fragment from S₁ digestion. The appearance of several bands at that region probably results from S₁ nibbling at the end. Figure 3 shows that no rat MLC2 transcripts could be detected in skeletal musIce RNA or any of the other seven tissues of mouse MLC₁. In mouse MLC₂, however, transcripts were detected in skeletal muscle RNA but not in RNA from the other tissues tested. Analysis of RNA prepared from mouse MLC₄ revealed the presence of a large amount of the rat transcripts only in skeletal muscle (data not shown).

Muscle-specific transcripts in transgenic mice MLC₂ and MLC₄ could arise either from readthrough transcription initiated from upstream mouse promoter expressed in skeletal muscle or from initiation at the authentic rat MLC2 promoter. To distinguish between these two possibilities, the site of the initiation of these transcripts was determined. The coding strand of a 289-bp *Sau*3A/*Eco*RI DNA fragment ³²P-labelled with T4 polynucleotide kinase was hybridized to RNA from differentiated cultures of the rat myogenic cell line L8, to control mouse skeletal muscle RNA and to skeletal muscle RNA from males and females of the first generation progeny of mice MLC₁, MLC₂ and MLC₄. RNA from the rat myogenic cell line protected a 50-nucleotide DNA fragment (Fig. 4). The appearance of two bands spaced one nucleotide apart probably results from S₁ nibbling of the sequence that immediately precedes the cap site. RNA from control mouse skeletal muscle protected a 76-nucleotide DNA fragment. These unexpected results suggest that: (1) there is a similarity in the sequences of the 5'-untranslated exon and immediate flanking region of the rat and the mouse MLC2 genes and (2) the transcription of the mouse MLC2 gene is initiated ~20 nucleotides 5' to the initiation site of the rat MLC2 gene. In more stringent hybridization conditions, such as those

chosen for the *Hin*fl DNA fragment (Fig. 3), there was no cross-homology with the mouse exon. (Also, compare the intensity of the signals of the 76-nucleotide band in Fig. 4*a*, *b*.) The stringency was lowered here so that the hybridization to the mouse exon is an internal control for the intactness of the RNA preparations of mouse MLC₁ and the control mouse. RNA from three out of four progeny of transgenic mouse MLC₂ and two progeny of mouse MLC₄ protected the diagnostic 50-nucleotide DNA fragment (Fig. 4). As expected, skeletal muscle RNA of MLC₁ mice gave no detectable signal.

The MLC2 gene is a member of a large group of genes activated during terminal differentiation of muscle cells[17]. The results presented here demonstrate that in two out of the three transgenic mice tested, the injected rat MLC2 gene responded to normal developmental controls. Its expression was detected only in skeletal muscle. Thus, the 4.7-kb rat DNA fragment containing the entire MLC2 gene includes *cis*-acting sequences sufficient to specify the correct expression of this gene. RNA transcripts of this gene were found in skeletal muscle in three of four progeny of MLC₂ and in two progeny of MLC₄, and not in any other tissue. The RNA had the correct initiation site of the authentic rat MLC2 gene, which differs from the endogenous mouse MLC2 by ~26 nucleotides. Interestingly, a similar frequency of non-expressing progeny (two of eight) has been reported for transgenic mice expressing the rabbit β-globin gene in skeletal muscle[7].

The levels of expression of the inserted rat gene are ~5-10% (in MLC₂) and 5-10× greater (in MLC₄) than in differentiated cultures of the rat myogenic cell line L8. The variable levels of specific expression could result from position effect, thus integration into non-homologous chromosomal sites may influence the level of expression of the foreign gene.

Several genes have been inserted into mice[1-15]. Tissue-specific expression of introduced genes, however, has been reported

only in four other cases; the rearranged immunoglobulin in κ gene[12], the rearranged immunoglobulin heavy-chain gene[15], the rat pancreatic elastase I gene[14] and the fused gene consisting of the 5' end of the mouse β-globin gene spliced to the 3' end of the human β-globin gene (F. D. Constantini, personal communication). In the first three cases and in the rat MLC2 gene in mouse MLC₄, the level of expression was similar to that of the endogenous gene, whereas in the globin and rat MLC2 genes in mouse MLC₂, the level of expression was considerably lower than the endogenous counterparts. One possible interpretation is that the immunoglobulin and the elastase I genes include tissue-specific enhancer sequences that are recognized as such even when the genes are integrated at different chromosomal sites. Evidence for the dramatic effects of enhancer sequences on the efficiency of expression of genes introduced into somatic cells has been obtained[18]. There is no evidence that the injected MLC2 DNA fragment includes specific enhancer elements. Therefore, the hundredfold difference in the level of expression in the two transgenic mice could result from either the absence of such elements or their weakness. Thus, in mouse MLC₂, these

sequences are neutralized easily by the local chromosomal environment at the site of integration, whereas in mouse MLC₄ they overcome the position effect.

Production of transgenic mice expressing muscle-specific genes or specifically modified genes in the appropriate tissue would allow a detailed study of the parameters affecting tissue-specific gene expression (for example, site of integration, DNA methylation or chromatin organization). Moreover, the control of expression of the inserted genes can now be analysed in myogenic and non-myogenic cells isolated from these mice.

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Regulation of a collagen gene promoter by the product of viral mos oncogene

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Oncogenic transformation of cells produces important changes in the biosynthetic pattern of certain cellular proteins[1-6]. For example, the synthesis of type I collagen in transformed fibroblasts is severely reduced as a result of changes in transcription[7-10]. Here we report the results of DNA-mediated transfection experiments using recombinant plasmids in which the promoter region of the α2(I) collagen gene is fused to an easily recognizable marker gene, and cell lines expressing the marker gene are isolated. Our data show that the expression of the marker gene fused to the cloned α2(I) collagen promoter is strongly inhibited by v-mos transformation, suggesting that a common mechanism inhibits both the transfected and endogenous α2(I) collagen promoters.

The α2(I) collagen gene is very large (~40 kilobase pairs(bp) with ~50 exons)[11] and is therefore difficult to manipulate in vitro. To overcome this problem in studying its expression by DNA-mediated transfection experiments, we have fused the promoter region of this gene to either the bacterial gene for chloramphenicol acetyltransferase (cat)[12] or the gene for aminoglycoside phosphotransferase (neo)[13]. Although such constructions may not contain all the regulatory elements controlling the expression of the endogenous α2(I) collagen gene, they do allow us to test whether the 5'-flanking sequences of the α2(I) collagen gene which are present in these hybrid plasmids are sufficient for regulation by oncogenes. Figure 1 shows the plasmids constructed for our experiments. We first transfected NIH 3T3 cells with DNA of plasmid pHO1000, in which the chick α2(I) collagen promoter is fused to the cat gene. This plasmid also contains the gene for guanine phosphoribosyltransferase

(gpt) fused to the early promoter of simian virus 40 (SV40). Clones positive for the expression of both the gpt and cat genes (AT1, AT2, AT3, etc.) were isolated. One of these clones (AT3) was examined by Southern blotting to show that the plasmid was stably integrated throughout at least two cycles of subcloning. We estimated that this cell line contains about eight copies of the α2(I) collagen promoter-cat DNA unit (data not shown). A Northern hybridization experiment indicated that the RNA produced by the collagen promoter-cat transcription unit in these cells was a discrete species ~1,600 nucleotides (Fig. 2a), consistent with a transcript starting from within the cloned chicken α2(I) collagen promoter and ending at the SV40 polyadenylation site present downstream from the cat gene in pHO1000. The results of a primer extension experiment (Fig. 2b) further indicate that the cat gene transcript starts at the correct site in the cloned α2(I) collagen promoter. In the same cells, gpt transcripts initiate at the major transcription start site in the early SV40 promoter (Fig. 2b, lane 2).

To determine whether the expression of the hybrid α2(I) collagen promoter-cat transcription unit was also inhibited by oncogenic transformation after it had been introduced into NIH 3T3, we first transformed three cell lines with the mouse Moloney sarcoma and leukaemia virus complex (MMSV). In all three clones, the level of CAT activity was reduced 7-14-fold after transformation (Fig. 3a, Table 1a). Dot-blot hybridizations using RNA extracted from one of these lines showed that the levels of endogenous α2(I) collagen RNA and those of cat RNA decreased 8-10-fold after MMSV transformation, whereas the levels of actin RNA were unchanged (Fig. 3b).

The plasmid construction used in these experiments also harbours the early promoter of SV40, which is fused to the bacterial gpt gene (Fig. 1a); this SV40 promoter segment includes the SV40 enhancer sequences[14,15]. To determine whether SV40 enhancer sequences are required for inhibition of the α2(I) collagen promoter after MMSV transformation, NIH 3T3 cells were transfected with pAZ1005, a plasmid in which a mouse α2(I) collagen promoter segment is fused to the neo gene but which does not contain SV40 enhancer sequences (Fig. 1c). Several colonies resistant to the antibiotic G418 (positive for the expression of the neo gene) were isolated and transformed either by MMSV or by a plasmid containing the

A Standardized Protocol for Assessing Regulators of Pigmentation

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Varied effects of chemical or biological compounds on mammalian pigmentation have been reported by many groups, but to date, no standardized method has established necessary and/or optimal parameters for testing such agents. A standardized method has been developed to screen compounds with potential effects on pigmentation. The protocol comprises basic parameters to analyze melanogenic effects and allows for further characterization of candidate compounds, providing important insights into their mechanism of action. In this protocol (termed STOPR, for standardized testing of pigmentation regulators), compounds are initially screened using purified tyrosinase and are then tested on melanocytes in culture. After treatment of melanocytes with potentially bioactive compounds, cell proliferation and viability, total melanin accumulated, and melanogenic potential are measured. This protocol is an important first step in characterizing chemical regulation of effects on melanogenesis. When bioactive candidate compounds are identified, testing may proceed for pharmacological or otherwise commercial applications in coculture and/or organ culture models followed by *in vivo* testing. As an application of this method, results for compounds known to stimulate and/or inhibit melanogenesis (including arbutin, hydroquinone, kojic acid, melanocyte-stimulating hormone, and thymidine dimers) as well as some commercial skin whiteners are reported.

Key Words: melanin; pigmentation; assay; tyrosinase.

Pigmentation in mammals is important to numerous distinct and critical processes, as illustrated by its ap-

pearance in various tissues of the body. Despite recent breakthroughs in the characterization of genetic and biochemical determinants of pigmentation, much remains unknown about the role of melanin in embryonic development, its function(s) in certain parts of the brain, eyes, and ears, its photoprotective nature in the skin, and its determination of phenotypic appearance. Skin and hair color depends on the amount, size, and type of melanins produced by melanocytes, pigment-producing cells found at the epidermal–dermal junction and in hair follicles (reviewed in 1–5). Starting with the enzyme tyrosinase, and in concert with other melanogenic enzymes, melanin is synthesized in melanosomes, is transferred from melanocytes to keratinocytes, and eventually disappears with desquamation of the skin and/or hair growth. Melanin is also an important defense of human skin against the harmful effects of UV light due to its ability to absorb and reflect UV energy, and its ability to scavenge oxidative free radicals (6–10). There is a dramatic inverse correlation between skin pigmentation and incidence of skin malignancies and UV photodamage (11–15). Pigmentation in humans also has an important cosmetic role which can be compromised in certain hyperpigmentary skin conditions and/or in lesions such as melasma, age spots, and postinflammatory hyperpigmentation (16–19).

There has been great variation in studies on various bioactive compounds targeted at regulating melanin production; in part, this variation stems from diverse conditions of melanogenic assay, different cell lines (including melanoma cells) used as targets, and different end points used to establish efficacy (20–23). In an attempt to standardize the assessment of novel bioactive agents for therapy of pigmentary lesions, a method has been developed which allows extensive testing of putative bioactives with minimal expense and effort, which renders outstanding reproducibility, and which accurately determines efficacy of action. In the

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STOPR[2] (standardized testing of pigmentary regulators) protocol, putative melanogenic regulatory compounds are initially screened using purified tyrosinase and are then tested for effects on cell proliferation, total melanin accumulation, and melanogenic potential using cultured pigmented murine melanocytes. The results obtained with the STOPR method show that this is a reproducible, economical, and reliable series of assays for screening potential melanogenic compounds, which provides important insights into the mechanism(s) of their action. Here we report the effects of melanogenic inhibitors (kojic acid, hydroquinone, arbutin, and niacinamide) and stimulators (α-melanocyte, stimulating hormone (MSH) and thymidine dimers), as well as some commercial formulations which contain putative inhibitory ingredients.

MATERIALS AND METHODS

Chemicals

All tissue culture medium components were obtained from GIBCO BRL (Long Island, NY). Propylene glycol, L-tyrosine, L-3,4-dihydroxyphenylalanine (Dopa), MSH, dimethyl sulfoxide (DMSO), hydroquinone, kojic acid, arbutin, niacinamide, phorbol 12-myristate 13-acetate (PMA), and β-mercaptoethanol were from Sigma Chemical Co. (St. Louis, MO). L-[U-^{14}C]Tyrosine was obtained from Dupont-New England Nuclear (Boston, MA). Mammalian tyrosinase used in the melanogenic assays was purified as previously described (24, 25).

The thymidine dimer mixture used was synthesized from thymidine (Sigma) by UV cross-linking with six 15-W lamps (254 nm wavelength) in distilled water at −78°C. The solvent was removed and the resulting oily residue was purified with a preparative gel filtration column using chloroform/methanol as a solvent. Five distinct fractions of thymidine dimers were isolated and identified (26) as pure dimers or as mixtures; only fraction 5, which consists of a mixture of the cis-syn and cis-anti dimers of thymidine was used in this study.

Compound Solubilization

Compounds were generally dissolved at 25 mg/ml in 25% PEH vehicle (12.5% propylene glycol, 7.5% ethanol) and diluted 25× in culture. Compounds that are not soluble in 25% PEH can be solubilized in modified solvents that contain increasing ethanol and decreasing propylene glycol (to a final concentration in culture

[footnote] Abbreviations used: DMSO, dimethyl sulfoxide; Dopa, 3,4-dihydroxyphenylalanine; MSH, α-melanocyte-stimulating hormone; EH, propylene glycol/ethanol/water solvent; PMA, phorbol 12-mystate 13-acetate; STOPR, standardized testing of pigmentary regulators.

of 2% ethanol or less) or in 2.5% DMSO (to a final concentration in culture of 0.1% or less). All compounds reported in this study were dissolved in 25% PEH, which was used as a solvent control. As an approach to determine solubility, 50 mg of each compound was dissolved in 1 ml 25% PEH. In case of poor solubility, 50 mg of the compound was dissolved in 1 ml DMSO and diluted with increasing amounts of H$_2$O to reach a final DMSO concentration of 2.5%. The compound concentration was then adjusted to 25 mg/ml in 25% PEH and solubility in both vehicles was compared. If compounds were not soluble in PEH or DMSO, the ethanol concentration was increased.

From stock solutions of 25 mg/ml, dilutions of 5 and 1 mg/ml were made with the selected vehicle, and aliquots of 200 μl were always added to the culture wells with 4.8 ml medium (i.e., a 25× dilution). In some cases, compounds were not fully soluble at the highest concentration in any vehicle tested, in which case a homogeneous suspension was used to prepare the next dilution, which was in most cases soluble. Compounds were tested at concentrations ranging from 0.06 μg/ml (for the most insoluble and/or cytotoxic compounds) to 1 mg/ml, at least in duplicate, as detailed in the figure legends.

Cells, Culture Techniques, and Treatments

Melan-a melanocytes were a kind gift of Dr. D. C. Bennett (St. George's Hospital, London). They were originally derived from C57BL/6 mice (27), and were grown in a humidified atmosphere with 10% CO$_2$ at 37°C. Cells were routinely passaged in complete RPMI 1640 medium, which contains 5% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM β-mercaptoethanol, 2 mM L-glutamine, and 200 nM PMA. Cells were harvested by brief treatment with trypsin/EDTA (GIBCO) and resuspended in complete RPMI 1640 medium. Viable cells, determined by trypan blue exclusion, were counted in a hemocytometer, resuspended in the appropriate volume of complete RPMI 1640 medium, and seeded at ~2 × 10^4 cells/well in 6-well plates.

For treatment of cells, compounds were added 24 h after seeding in duplicate wells at three different concentrations, as detailed in the figure legends. Equivalent volumes and concentrations of diluents were added to duplicate control wells. Cells were treated with compounds for a total of 4 days. After allowing 1 day for cell attachment, the medium was changed and compounds were added in fresh complete RPMI 1640 medium. Two days later, the medium was changed and fresh compounds were added in fresh complete RPMI 1640 medium. Two days later, cells were photographed and then harvested with 0.5 ml trypsin/EDTA for 3 min. After dislodging the cells with occasional agita-

tion, 2 ml of fresh complete RPMI 1640 medium was added to inactivate the trypsin and 100-μl aliquots were seeded into flat-bottom 96-well plates for the MTT assay, as described below. The remainder of the cell suspensions were centrifuged for 5 min at 1500g, washed with Dulbecco's phosphate buffered saline (without Ca and Mg), and then solubilized in 240 μl of extraction buffer (1% Nonidet P40, 0.01% SDS, 0.1 M Tris:HCl, pH 7.2, 1 μg/ml aprotinin, 100 μM phenyl-methylsulfonyl fluoride and Protease Inhibitor cocktail (Boehringer-Mannheim, Indianapolis, IN)). Extracts were solubilized at 4°C for 60 min and assays as described below were conducted for each sample, at least in duplicate.

Cell Viability and Proliferation

The MTT assay kit (Boehringer) was used to determine cellular viability and proliferation. Cells were treated for 4 days as described above and 100-μl aliquots of harvested cells were plated in flat-bottom 96-well microtiter plates. Cells were allowed to attach and grow overnight at 37°C before performing the MTT assay according to the manufacturer's instructions. The formazan precipitates were quantitated by absorbance at 562 nm in a SpectraMax 250 ELISA reader (Molecular Devices, Sunnyvale, CA) with a reference wavelength of 690 nm. Absorbance values (562–690 nm) of controls and treated cells were measured. The doubling time for melan-a melanocytes is typically 48 h, thus two cell doublings occur during the 4-day treatment period. The number of cells (% confluence) was observed under an inverted phase-contrast microscope in three randomly selected fields of view. Percentage confluence was assessed before harvesting and again before performing the MTT assay.

Melanin Content in Cell Extracts

Melanin content was measured using a modification of a previously reported method (28). Aliquots of extracts (100 μl) were transferred to 96-well plates, mixed well, and immediately quantitated by absorbance at 650 and 490 nm. Melanin contents of control and treated samples are reported on a per well basis.

Radiometric Assays

Compounds were tested for direct effects on tyrosinase activity using a modified radiometric tyrosinase assay (24, 25), and the same assay was used for measuring effects on melanocytes. Compounds were solubilized at an initial concentration of 5 mg/ml. Briefly, the melanogenic tyrosinase assay was performed in quadruplicate in 96-well microtiter plates by adding 10 μl compound and 20 μl purified tyrosinase, in that order. After 30 min preincubation at 23°C, 10 μl of

L-[^{14}C] tyrosine was added along with 10 μl 0.25 mM L-Dopa cofactor in 1 M sodium phosphate buffer, pH 7.2, containing 0.01% albumin. Reactions were incubated for 1 h at 37°C, after which 100 μl 0.1 M HCl with excess unlabeled L-tyrosine was added to each well. The contents of each well were removed with a multichannel pipettor to a dot-blot apparatus (Bio-Rad, Hercules, CA) and acid-insoluble radioactive melanin and melanin precursors were bound to ZetaProbe blotting membranes (Pharmacia, Piscataway, NJ) for 15 min at 23°C. The membranes were then dried under vacuum and washed three times with 250 μl 0.1 M HCl with excess unlabeled tyrosine; they were then removed from the apparatus and washed three more times for 20 min each with 100 ml 0.1 M HCl. Membranes were then air-dried and exposed to a Storm phosphor screen; quantitation of radioactive melanin production on those blots was performed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

Visible melanin on those same blots was quantitated by two methods: (a) Membranes were photographed with a Kodak DC120 digital camera, and individual spots on the images were analyzed by computer using the NIH Image program, available at http://rsb.info.nih.gov/nih-image/default.html; and (b) membranes were also quantitated with a Minolta ChromaMeter CR-241; three random fields were measured for each spot and the three color parameters (L*, a*, and b*) were converted to percentage control. In the L* a* b* color space coordinates, L* indicates lightness, and a* and b* are the chromaticity coordinates; +a* is the red direction, -a* is the green direction, +b* is the yellow direction, and -b* is the blue direction. The center is achromatic, +L* is white and -L* is black.

Miscellaneous

Protein concentrations were determined with the BCA assay kit (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the standard, following the manufacturer's instructions for the microtiter plate format.

RESULTS AND DISCUSSION

General Considerations

Many potential approaches for testing melanogenic effects were considered in optimizing the STOPR method to accurately, reproducibly, and economically measure inhibition or stimulation of melanogenic activity. Screening for bioactive melanogenic compounds requires four discrete steps, quantitating (1) effects on enzymatic activity *in vitro*, (2) effects on cultured normal melanocytes, (3) effects on melanocyte:keratinocyte cocultures, and

A) Visible melanin - detected by eye
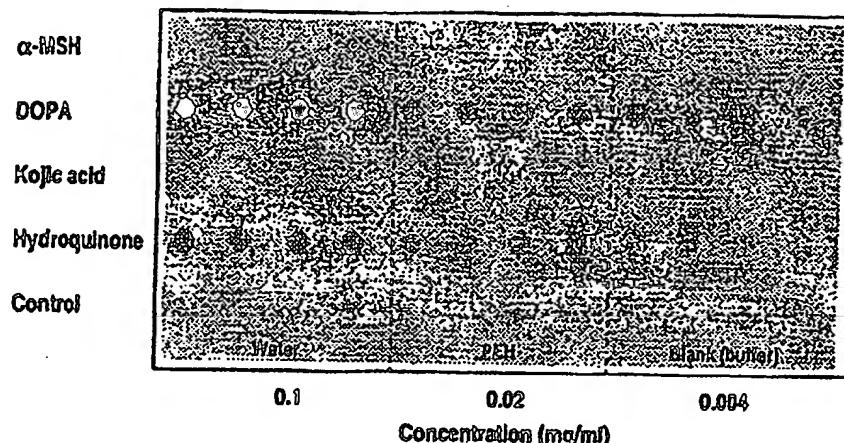
**B) Radiolabeled Melanin - detected by autoradiography** ·
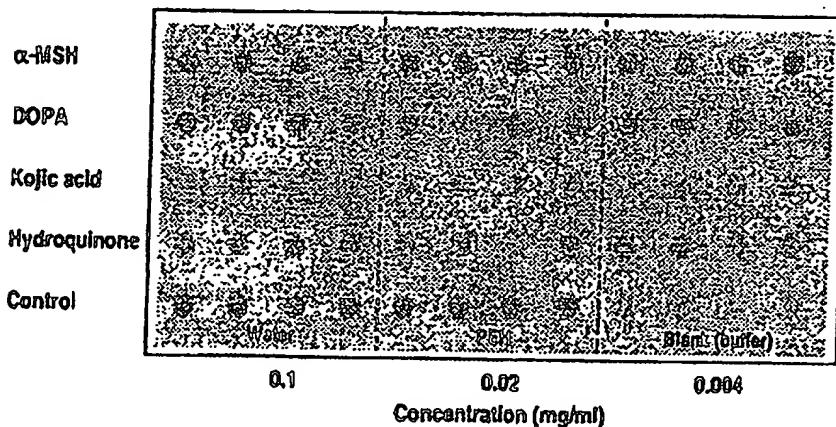
FIG. 1. *In vitro* dot-blot radiometric assay of selected melanogenic compounds. Purified tyrosinase was preincubated with the compounds noted on the left at the indicated concentrations and then assayed in quadruplicate for tyrosinase activity using the [^{14}C]tyrosine dot-blot assay on a ZetaProbe membrane, as detailed under Materials and Methods. (A) Dot-blot membrane photographed with a Kodak DC120 digital camera to show visible melanin. (B) The same membrane scanned with a Storm PhosphorImager to show radioactive melanin. (C) Quantitation of visible melanin shown in A generated with the software program NIH Image; results are expressed as mean gray level (control = 1% PEH). (D) Quantitation of radioactive melanin shown in B assessed with ImageQuant software; results are expressed as volume × 10^{-3} (control = 1% PEH). (E) Measurement of visible melanin shown in A measured with a Minolta ChromaMeter CR-241; L° (light/dark), a° (red/green), and b° (yellow/blue) color parameters, as discussed in the text, are quantitated and are expressed as % control (1% PEH). % SE in all results shown are less than ±5%.

inally, (4) toxicity and efficacy testing *in vivo*. This study focused on methodology for the first two steps, which are critical to initial screening of putative bioactive compounds. Primary melanocytes in culture might be thought to be optimal to examine *in vivo* effects, but in reality, due to the limited proliferative capacity of primary melanocytes and the variability of phenotypes generated, immortalized melanocytes, such as melan-a, are a more reliable

model. Pigmented melanoma cell lines have often been used in previous studies to assess the efficacy of melanogenic compounds (21, 29–37), but transformed cells are really not appropriate models for epidermal hypo- or hyperpigmentation since they are abnormal melanocytes by definition, and since their proliferative potential and disrupted intracellular signaling interferes with normal melanin production (cf. discussion below). Pigmented melan-a melano-

C) Visible melanin - quantitated by densitometry
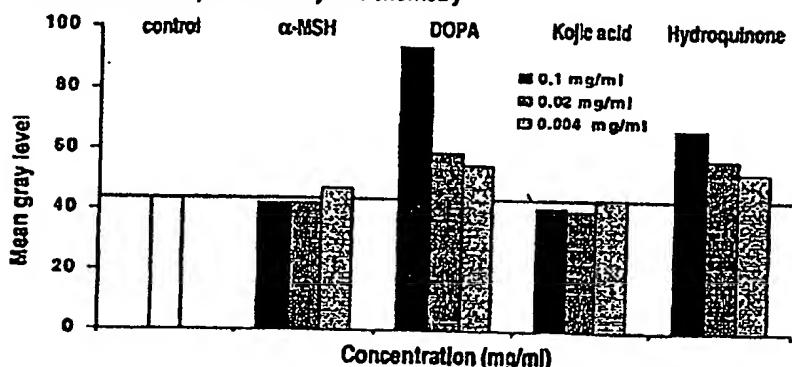
D) Radioactive melanin - quantitated by ImageQuant
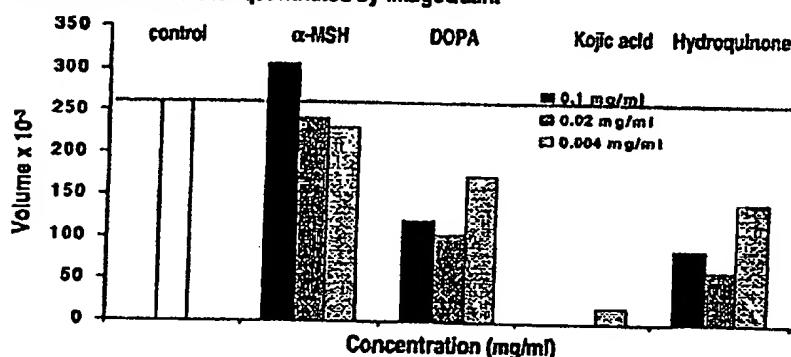
## E) Visible melanin - quantitated by ChromaMeter
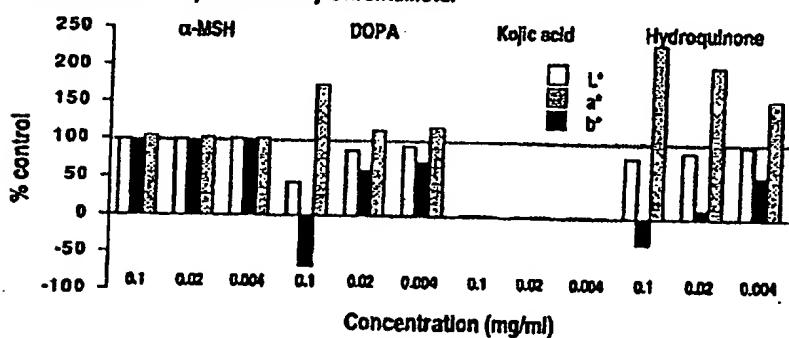
FIG. 1—Continued

ytes are routinely used for testing in this protocol; these melanocytes are pigmented and dendritic and are only slowly proliferative (27). It should be noted that PMA is routinely added to all media since it is required for melanocyte proliferation. However, PMA depletes PKC and thereby at least in part utes intracellular signaling events and can decrease melanocyte responsiveness.

In Vitro Testing

Compounds were initially tested *in vitro* on purified tyrosinase, using a [^{14}C]tyrosine dot-blot assay to quantitate effects of bioactive compounds on melanin content and melanogenic enzyme activity. Initially, the affinity of different membranes for radiolabeled melanin was assessed, including 3MM filter papers (What-

man, UK) which were used in the originally described assay (24, 25), nitrocellulose, Nytran and ZetaProbe membranes. ZetaProbe filters resulted in the highest affinity with the lowest background (data not shown) and were used in all subsequent experiments, although the other membranes could be used suboptimally. To characterize these assays, compounds examined included those known to stimulate melanogenesis (e.g., Dopa and MSH (38)) or to inhibit melanogenic function (e.g., arbutin, hydroquinone and kojic acid (39–42)).

Compounds were titrated for effects on tyrosinase using the [^{14}C]tyrosine melanogenic assay, as detailed under Materials and Methods. Figure 1A shows the appearance of the ZetaProbe membrane after binding and washing of the samples. Radioactive melanin on the same membrane as detected by PhosphorImager is shown in Fig. 1B. The amount of visible melanin was readily quantitated by membrane scanning and analysis with NIH Image software (Fig. 1C) or with a ChromaMeter (Fig. 1E); radioactive melanin formed was quantitated with a Storm 860 PhosphorImager and ImageQuant software (Fig. 1D). Production of melanin (either visible or radioactive) in response to various compounds is readily appreciated when compared with the three sets of controls on the bottom row of the membrane. Controls in the bottom row contained enzyme in aqueous solvent (the group of four on the left), in PEH vehicle (the group of four in the middle), or no enzyme (the group of four on the right) to establish background. Note that there is generally, but not always, a direct correlation in visible and radioactive melanin produced. As an example, preincubation of tyrosinase with the inhibitor kojic acid led to comparable decreases in visible and radiolabeled melanin formation, whereas αMSH had no effect on enzyme activity using either criterion (as expected since this hormone works through a melanocyte surface receptor). In contrast, incubation of the enzyme with Dopa or hydroquinone, which are alternative substrates for tyrosinase, led to dramatic increases in visible melanin production (Figs. 1A, 1C, and 1E), but resulted in decreases in radioactive melanin production (Figs. 1B and 1D). This result is to be expected since Dopa is unlabeled, and melanin production using Dopa as a substrate is increased. At the same time, radioactive melanin production was actually decreased since Dopa competed with the [^{14}C]tyrosine substrate. With hydroquinone, the rationale is similar: oxidized hydroquinone is analogous to the early quinone products of tyrosinase that later become high molecular weight melanins. Hydroquinone would increase visible melanin formation but, since it is not labeled, radioactive melanin production was not increased. Further, quinones may inactivate tyrosinase by a suicide inactivation mechanism (43) which is consistent with the observed decrease in activity, and quinones have been

shown to inhibit a number of other enzymes (44). Hydroquinone can also act as an antioxidant, as discussed below. Note that different compounds gave rise to different colors of melanin, as reflected in the different a° and b° parameters for Dopa and hydroquinone assays measured by ChromaMeter (Fig. 1E). The color of Dopa–melanin was dark brown to gray, whereas that of hydroquinone–melanin was a shade of orange-brown, compared to controls.

These compounds gave comparable results when tested for effects on melanogenic activity in soluble extracts of melan-a cells (data not shown). Such crude extracts of melanocytes are probably generally sufficient to use for screening effects on tyrosinase activity if purified tyrosinase is not available. Most certainly, mammalian tyrosinase should be used for these studies, not commercially available mushroom tyrosinase, because of the dramatically different substrate and cofactor requirements, as well as sensitivity to inhibitors (21, 45–47).

Since tyrosinase is the rate-limiting enzyme of melanogenesis, the most logical regulatory point to control pigment formation is by inhibiting (or stimulating) that activity. Indeed, this has been the approach generally used in commercial formulations aimed at lightening skin color. The STOPR protocol has been used to examine the efficacy of several of these commercially available products and their active ingredients (Fig. 2). Whitess, Anti-Taches Mains, and Fair & Lovely were tested, as were their active ingredients, arbutin, kojic acid, and niacinamide, respectively. Arbutin was inhibitory toward melanin production in a dose-dependent manner and this corresponded well with the inhibition observed with the commercial product containing it. Kojic acid inhibited radioactive melanin formation even more dramatically, as did the commercial product containing it. Niacinamide (also known as 3-pyridinecarboxamide, niacin, and vitamin B$_3$), has been found to be an antioxidant by a free radical scavenger mechanism (48, 49). In our assays, niacinamide itself did not show much inhibition of melanogenic activity at the concentrations tested, although the commercial formulation (with an unspecified niacinamide concentration) did inhibit melanogenic activity in vitro, which suggests the possibility that there is another active ingredient or that niacinamide was more active or stable in that product. Thus, we conclude that the STOPR assay is an appropriate way to screen commercial products for efficacy, as well as their bioactive ingredients.

Testing of Compounds in Tissue Culture

Once inhibitory or stimulatory effects on melanogenesis have been established and active compounds have been identified, the effects of those compounds on melanocytes in culture are tested. After treatment of

A) Radioactive melanin

control   Arbutin   Kojic acid   Niacinamide

400
350
300
250
200
150
100
50
0
-50

Volume x 10⁻³

■ 1 mg/ml
▨ 0.2 mg/ml
▨ 0.04 mg/ml
□ 0.008 mg/ml

Concentration (mg/ml)

B) Radioactive melanin

control   Shiseido Whitess (Arbutin)   Vichy anti-taches Mains (Kojic acid)   Pond's Fair & Lovely (Niacinamide)

400
350
300
250
200
150
100
50
0
-50

Volume x 10⁻³

■ 1 mg/ml
▨ 0.2 mg/ml
▨ 0.04 mg/ml
▨ 0.008 mg/ml

Concentration (mg/ml)
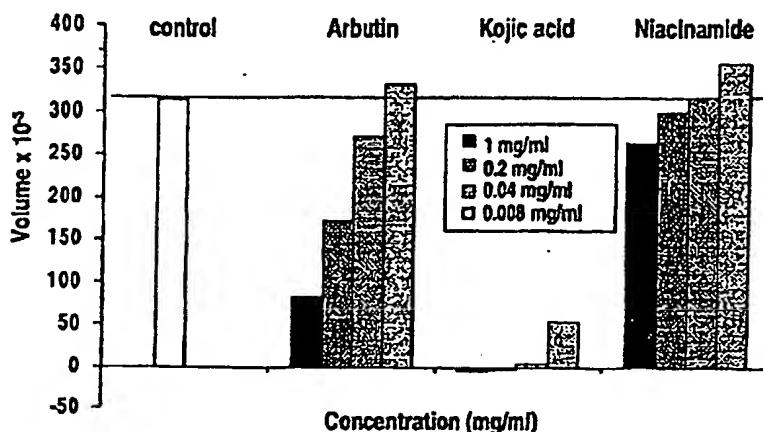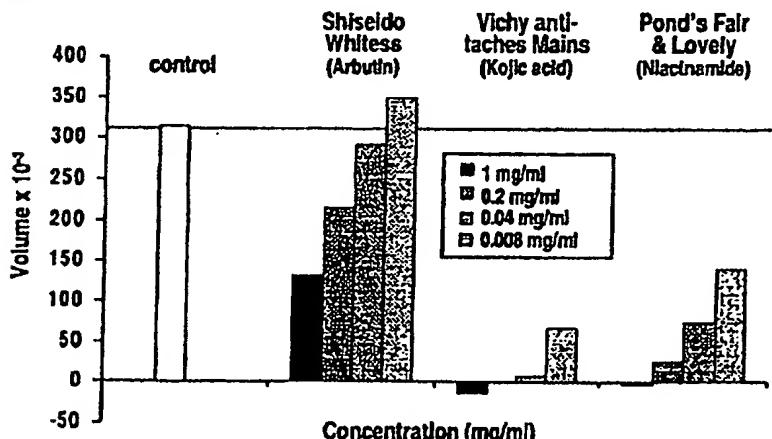
FIG. 2. *In vitro* dot-blot radiometric assay of selected commercial formulations. Commercial skin whiteners and their active ingredients were assayed *in vitro* on purified tyrosinase, in quadruplicate, as detailed for Fig. 1: quantitation of dot blots is shown in these graphs and is reported as volume X·10⁻³ (control = 1% PEH).

melan-a melanocytes with various compounds for 4 days, cells are harvested and four different parameters of cellular function are measured by microassay, namely, effects on growth, on viability, on melanin content, and on melanin synthesis, as shown in Fig. 3. Those assays are quantitated as detailed under Materials and Methods and are plotted in Fig. 4.

An important concept when selecting bioactive compounds that modulate skin pigmentation is that, for obvious reasons, they should have minimal effects on melanocyte proliferation and/or survival. In these experiments, effects of treatment on cell growth are noted visually (estimation of % conflunce) (Fig. 4A). Effects on cell proliferation are as-

sessed using the MTT assay (Figs. 3B and 4B), effects on melanin content are measured by absorbance at 650 nm (Figs. 3C and 4C), and effects on the melanogenic activity are measured as [¹⁴C]tyrosine incorporation into nascent insoluble melanin (Figs. 3E and 4D). As with purified enzyme, visible melanin on the dot blots can be measured (Fig. 3D). Results of melanogenic assays are reported in PhosphorImager measurements as arbitrary volume units. Effects on the melanogenic enzyme activity of melanocytes can differ from visible pigmentation after 4 days of treatment, as discussed above. Further, melanogenic enzyme assays reflect direct effects of compounds on tyrosinase activity and/or other me-

VIRADOR ET AL.

A)
MSH
DOPA
Kojic acid
Hydroquinone
Thimidine dimer
Control
St. curve

B)
MSH
DOPA
Kojic acid
Hydroquinone
Thimidine dimer
Control

C)
MSH
DOPA
Kojic acid
Hydroquinone
Thimidine dimer
Control

D)
MSH
DOPA
Kojic acid
Hydroquinone
Thimidine dimer
Control

E)
MSH
DOPA
Kojic acid
Hydroquinone
Thimidine dimer
Control
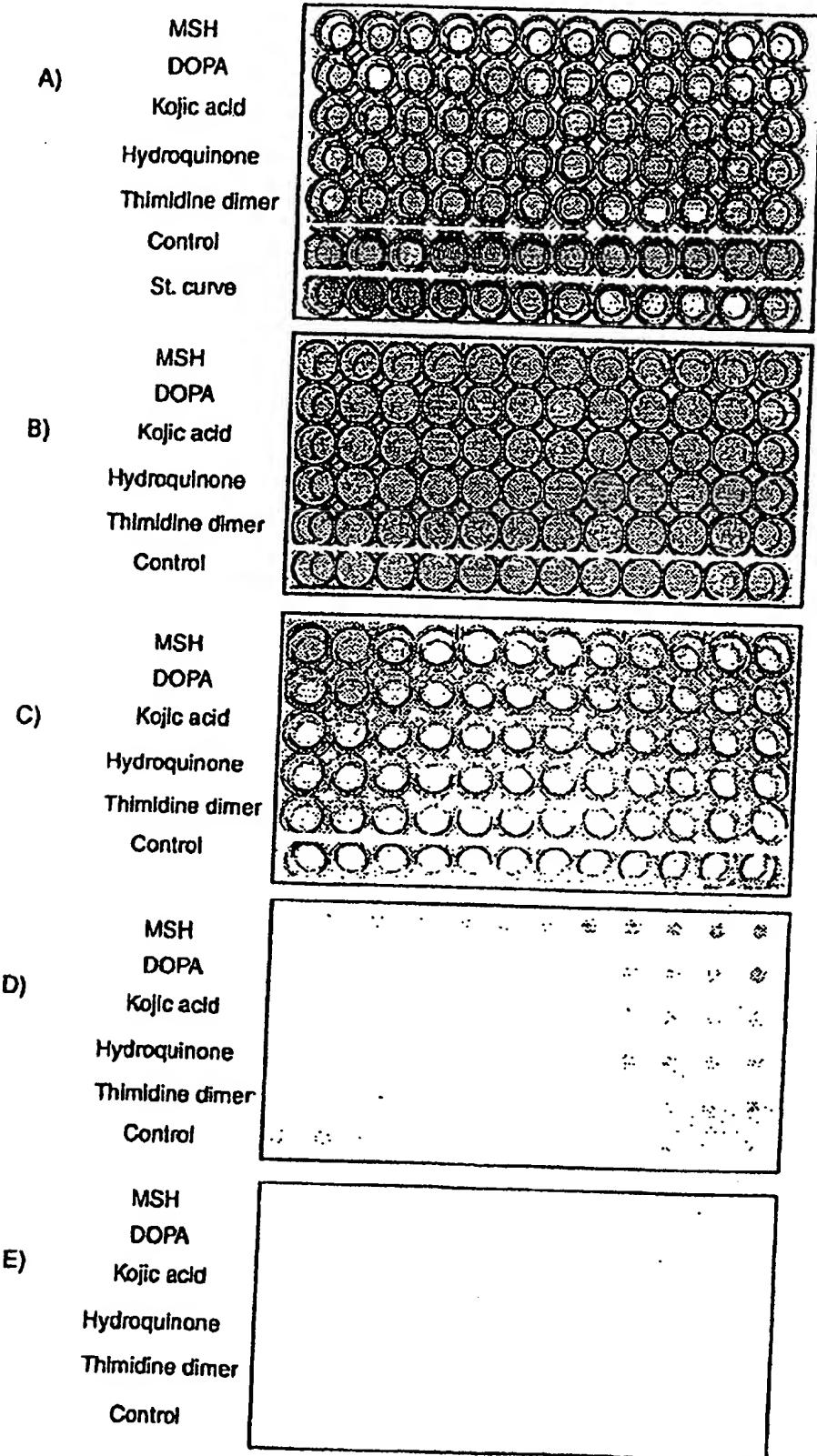
A) Cell viability
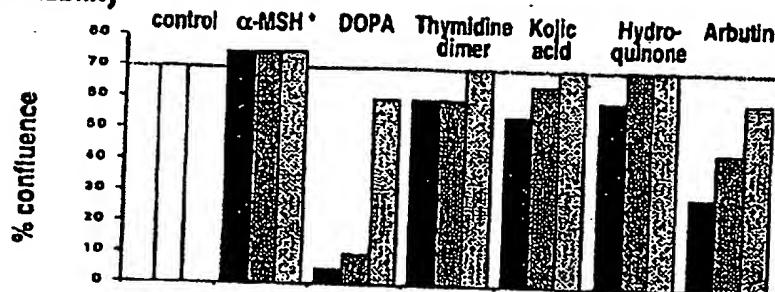
B) Cell proliferation
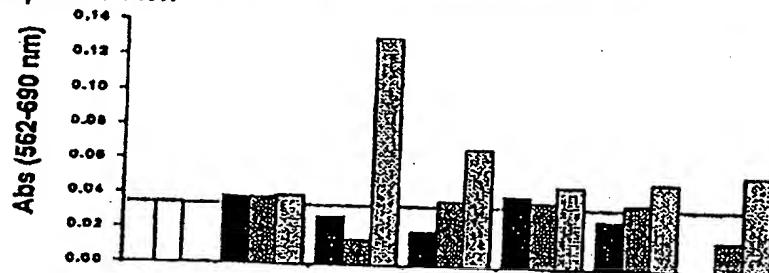
C) Total melanin

D) Radioactive melanin
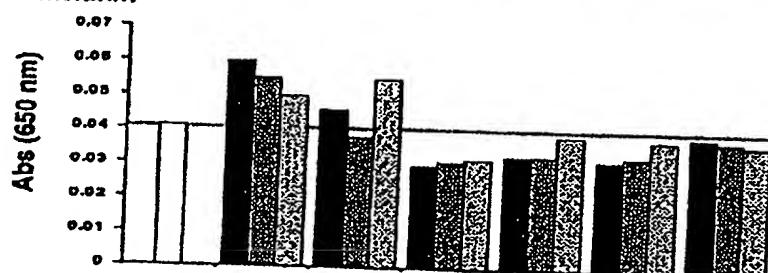
Concentration (mg/ml)
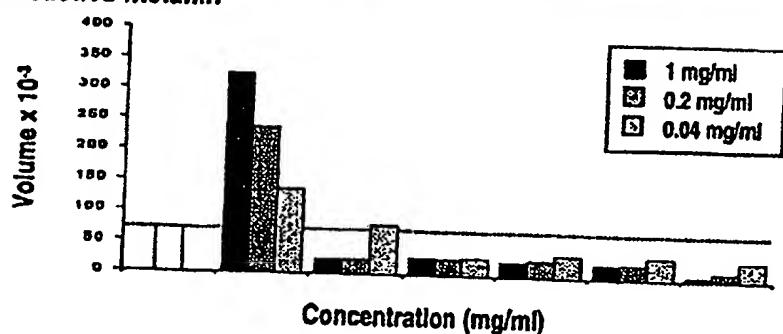
FIG. 4. Quantitative assays of treated melanocytes. Results of treatment of melan-a melanocytes with tyrosinase inhibitors or stimulators at the concentrations indicated in Fig. 3 are plotted. (A) Cell viability, expressed as % confluence of cells prior to harvest; (B) cell proliferation, measured by the MTT assay (from Fig. 3B); (C) total melanin content, measured by absorbance at 650 nm (from Fig. 3C); and (D) radioactive melanin formation, measured as volume × 10^{-3} (from Fig. 3E). Each value shown represents the mean of quadruplicate assays; SE were always less than ±5% of the mean.

FIG. 3. Effects of compounds on melanocytes in culture. Melan-a melanocytes were treated for 4 days with the compounds indicated, in quadruplicate, as described under Materials and Methods. Compounds were tested at 1.0 (left in each group). 0.2 (middle), and 0.04 mg/ml (right), except for α-MSH which was tested at 50, 10, and 2 nM, respectively. (A) Protein concentration of cell extracts, measured by the BCA assay; (B) cell viability and proliferation, measured by the MTT assay; (C) total melanin accumulated per well, measured as absorbance at 650 nm; (D) visible melanin (as shown in Fig. 1A); and (E) radioactive melanin (as shown in Fig. 1B).

A)

B)
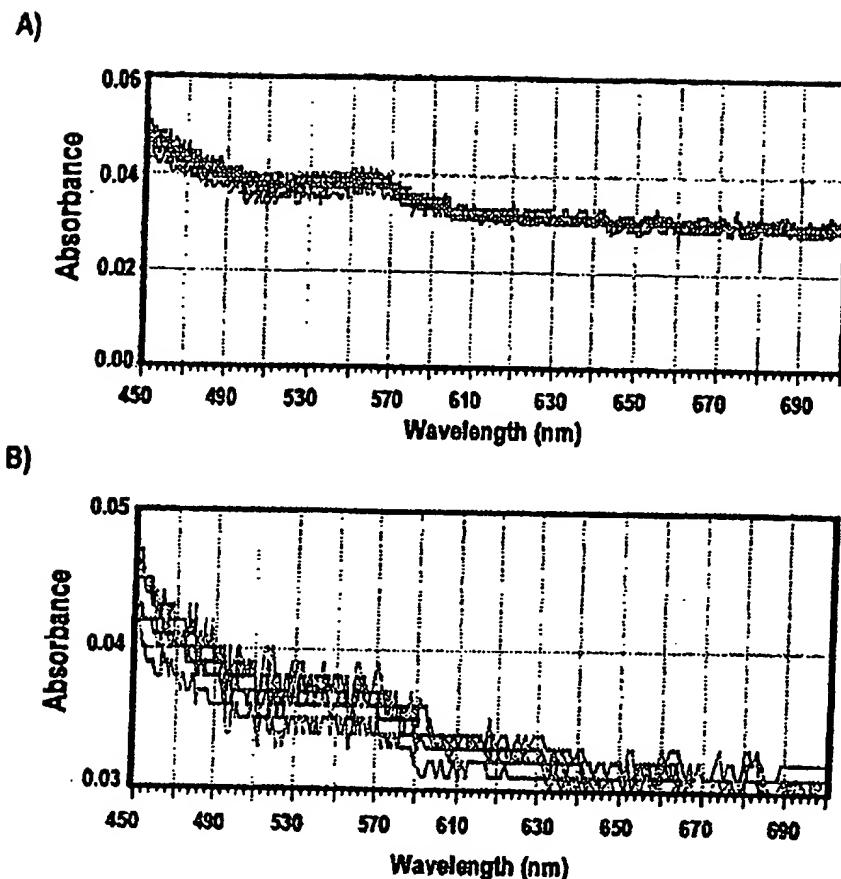
FIG. 5. Absorbance of melanin in cell extracts. Cell extracts from treated cells (in the format shown for Fig. 3D) were scanned in a microplate reader at wavelengths from 450 to 700 nm, at 1-nm intervals. The SoftmaxPro 2.4 program was used to generate overlays of different scans for analysis. (A) Scans of extracts from 12 wells, representing 3 different concentrations of arbutin-treated cells; (B) scans of extracts from cells treated with arbutin or with 4 other test compounds, as well as untreated controls, at 3× higher sensitivity. Note the relatively uniform absorbance of melanin across all wavelengths, but variation in readings is approximately twofold greater at 490 nm than at 650 nm.

lanogenic enzymes, while cellular melanin contents also reflect turnover, accumulation, and distribution of melanin associated with cellular proliferation.

In preliminary experiments, differences in melanin absorption at 490 and 650 nm were assessed since each wavelength has been suggested to be optimal in earlier studies (50). Although both wavelengths were in fact quite accurate in estimating melanin content, there was less variability at 650 nm than at 490 nm resulting from variability in the color of melanins produced in the samples (Fig. 5). Absorption at 650 nm seems to be the best compromise to assess melanin content under all conditions.

Compound solubility in tissue culture medium was sometimes a significant problem at higher concentrations. Commonly used vehicles were tested for cytotoxicity in culture, and PEH and DMSO at nontoxic concentrations were finally selected as optimal choices.

Consideration of compound solubility is an important criterion, since drug design coupled to crystallographic visualization of the target site can lead to the development of drug candidates with potent biological activity. However, many of these are subsequently found to have poor solubility, poor bioavailability, and/or to be extensively metabolized and inactivated. The choice of vehicle itself is also relevant; e.g., PEH itself has been shown to stimulate melanogenesis in Cloudman S91 mouse melanoma cells at concentrations of 100 mM and higher (51). In this study, a low concentration of PEH was used and no induction of melanogenesis in PEH controls above background was observed.

Three compounds that have been reported to stimulate melanocyte production of melanin were examined (MSH, Dopa, and thymidine dimers) as well as three putative inhibitory compounds (arbutin, kojic acid, and hydroquinone). Of the potential stimulatory agents

tested, MSH had a slight stimulatory effect on cell number estimated by percentage of confluence (Fig. 4A) and on cellular proliferative potential (Fig. 4B). Note that MSH was used at physiological concentrations (i.e., 50, 10, and 2 nM) rather than at the standard concentrations used for other compounds. MSH elicited a significant increase in melanin content (Fig. 4C) and an even more dramatic stimulation of melanogenic activity (Fig. 4D). Stimulation of proliferative potential by MSH has been previously reported (52).

Treatment with Dopa led to a dramatic decrease in cell number and proliferative potential, presumably due to the cytotoxicity associated with rapid increases in production of melanin (and toxic melanogenic intermediates), as previously reported (53, 54). Addition of Dopa, which can function as a substrate and as a cofactor for tyrosinase as noted above, to melanocytes elicited an increase in enzyme activity (Fig. 4D) and visible melanin formation (Fig. 4C) at the lowest concentration, as might be anticipated based on the results presented above. It has been suggested that Dopa can cause membrane damage and release of tyrosinase into the culture medium (55), causing cell leakage and reduction of protein levels.

Thymine dinucleotides have been reported to stimulate melanin production in vivo (56), and so the effect of thymidine dimers, the actual DNA photoproduct generated by UV, on melanocyte function was tested. Thymidine dimers slightly inhibited growth of melanocytes at higher concentrations (Figs. 4A and 4B) but led to a decrease in melanin content (Fig. 4C) and in melanogenic activity (Fig. 4D). It should be noted that stimulation of melanogenic activity and melanin production has occasionally been noted in some experiments, particularly when the data are corrected for protein contents, and further study will be necessary to characterize these variable effects.

Interestingly, the three inhibitors examined, arbutin, kojic acid, and hydroquinone, had significant inhibitory effects on melanocyte viability (Fig. 4A) and proliferation (Fig. 4B), particularly at the higher concentrations. All three compounds decreased total melanin content (Fig. 4C) and inhibited melanogenic enzyme activity (Fig. 4D). It should be noted that the data for melanin content and enzyme activity have not been corrected for protein content in the presentation of these results, and this may be a significant factor when there are significant effects on melanocyte growth. Protein content can be readily measured by the BCA assay as shown in Fig. 3A) and these data can be adjusted or that if desirable. Similarly, standard curves can be constructed with known melanin concentrations and known enzymatic activities so that data can be presented in whatever units are needed.

CONCLUSIONS AND IMPLICATIONS

Chemical compounds (synthetic or natural products) have been used by dermatologists to improve the cosmetic appearance of patients who have pigmentary skin disorders. Compounds such as hydroquinone are known to produce topical depigmentation, mostly due to their cytotoxic effects on melanocytes at sufficient concentrations. Destruction of melanocytes leads to permanent loss of constitutive pigmentation and the need to find reliable assays to develop and evaluate new active ingredients for cosmetic and/or pharmacological use is widely recognized. In the case of skin whiteners, a growing market makes it imperative to standardize procedures so that the safety and efficacy of new and existing compounds can be properly evaluated.

The STOPR protocol consists of two main steps: (1) testing of effects on melanogenesis in vitro, for which a simplified dot-blot melanogenesis assay was developed, and (2) testing of melanocytes in culture, for which a battery of additional tests in microtiter plate format was developed. Various modifications of the STOPR protocol have been tested over the past 2 years, and its current form has been streamlined and optimized for speed, economy, reproducibility, and accuracy. The total time required for culture assays per compound for an experienced worker is ~3 h (from weighing of compound to obtaining final data). The total cost per compound for the full series of assays would be $20 at current price levels. To maintain maximal reproducibility, special attention should be paid to: (1) melanocyte passage number (upon prolonged passage, cultured melanocytes tend to lose their pigmentation and their response to regulatory agents) and (2) cell density (plates are seeded at the same density and treated at standard times as noted during cell log phase because contact inhibition of melanocytes leads to cellular quiescence and spontaneous increases in melanin production).

Some assays used in the STOPR protocol are based on larger-scale assays for tyrosinase activity or melanin content that have been routinely utilized in compound screening (23, 39, 57, 58), while others have been adapted specifically for this purpose from our laboratory's expertise in the field. Each assay was modified to be compatible with the microtiter plate format, to streamline processing and to take advantage of automated equipment available to handle such testing format. For example, there is excellent agreement between results obtained with this new melanogenic assay and with the previously described melanogenic assay, which uses 3 MM filter papers and a scintillation counter to measure ^{14}C-labeled insoluble melanin. Simple PhosphorImager scanning of ZetaProbe blots can readily assess melanogenic effects on 96 samples in

a single blot, and the effects can be easily quantitated using a PhosphorImager if one is available. Either method can be implemented depending on instrument availability, but with the original technique, processing of 200 assays would require an entire work day. Using microtiter plates, productivity of the same worker can easily be increased fivefold, with significant reductions in number of cells, compounds, and radioactive materials required.

After screening of candidate compounds has been performed with the STOPR protocol, promising bioactives may be tested in a model system comprised of melanocytes/keratinocytes in coculture or in a synthetic skin model system since tissue homogenates cannot reflect transport of compounds through intact skin. We are currently investigating the feasibility of further functional testing of candidate compounds in such physiological skin models.

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a *HindIII/EcoRI* digest of pBluescript KS(−) (Stratagene) to give pTY811B (Fig. 1). In order to use the cDNA part of Tyrs-J, a 1.85 kb *XhoI/EcoRI* cDNA fragment from Tyrs-J (12) was used to replace the analogous cDNA region of MTY811H. Subsequently, the homologous 4.1 kb *HindIII-EcoRI* fragment was subcloned into pBluescript KS(−) to give pTyBS (Fig. 1). To generate chimeric constructs, pTyBS and pTY811B were both digested with *ScaI*. Both plasmids contain one *ScaI* site in the tyrosinase sequences and one in Bluescript. The 5′ tyrosinase sequences of pTY811B were ligated to the 3′ sequences from pTyBS to give pTY811D, and *vice versa* for pTY811C. The *ScaI* site in Bluescript is located within the amp' gene, allowing the desired orientation of ligation to be selected by culture on ampicillin plates. pTy811C has the Tyrs-J coding region at amino acid 103, while pTy811D has the MTY811c sequences (see Fig. 1). These coding regions were confirmed by double-stranded dideoxy sequencing. The *ScaI* ligation sites were also sequenced and shown not to contain any insertions, deletions, or base pair changes. Note that pTy811C is not the same as MTY811c.

Generation of transgenic mice

Transgenic mice were generated by the standard technique of microinjection into single cell mouse embryos (17). FVB/N female mice mated to FVB/N males were used as embryo donors. ICR females bred to vasectomized BDF1 males were used as embryo recipients and surrogate mothers.

Southern hybridizations

Tail DNAs from potential transgenic mice were isolated as described previously (17). DNAs of common laboratory mouse strains were purchased from Jackson Laboratory (Table 2, all strains except FVB/N and ICR/Hsd). Aliquots (10 μg) of genomic DNA were digested with *PstI* or *EcoRI*, then electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane (Zeta-probe, Bio-Rad). A 1.9 kb of *BglII-EcoRI* fragment of the mouse tyrosinase cDNA clone MTY811c (10) was labelled with ^{32}P-dCTP by the random primer method (18) and used as a hybridization probe. Hybridizations were done in 5× SSC, 5× Denhardt's and 45% formamide at 42°C overnight (1× SSC is 150 mM NaCl, 15 mM Na citrate). Hybridization membranes were washed to a final stringency of 0.1×SSC, 0.1% sodium dodecyl sulfate at 65°C.

Polymerase chain reaction and DNA sequencing

The first exon of the tyrosinase gene was amplified directly from genomic DNA by use of the polymerase chain reaction (PCR) (19) with oligonucleotide primers, 5′-GGGGTTGCTGGAAAA-GAAGTCTGTG-3′ (nucleotides −73 to −49 in tyrosinase exon 1) and 5′-AACTCTCTCTATATAGTGCATCTT-3′ (antisense to sequences of tyrosinase inton 1). Amplification conditions were: denaturation for 30 sec at 92°C, 1 min annealing 62°C and 2 min polymerization at 72°C for 30 cycles. The reaction products were diluted with an equal volume of H₂O and an equal volume of 7.5M ammonium acetate, followed by precipitation with 2.5 volumes of ethanol. After centrifugation, precipitates were washed in 75% ethanol, dried, and resuspended in 8 μl of H₂O. The sequencing primer 5′-TGCTAAAGTG-AGGTAAGAAAAGAAC-3′ (nucleotides 423 to 399 in tyrosinase exon 1) was end-labelled with γ³²P-ATP using polynucleotide kinase, then purified using an NENSORB™20 column as described by the manufacturer (New England Nuclear). PCR-amplified template DNA was alkali denatured, annealed

with the end-labelled primer, and subjected to dideoxy sequencing (Pharmacia ⁷⁷sequencing kit) using the manufacturer's protocol.

RESULTS

A cysteine to serine substitution at amino acid 103 inactivates tyrosinase minigenes

To investigate the importance of the amino acid differences between Tyrs-J and MTY811c, four different tyrosinase minigenes were made (TyBS, Ty811B, Ty811C and Ty811D) (see Fig. 1). All four constructs have the same 2.25 kb of upstream regulatory sequences including the first 65 base pairs of exon 1. Attached to this region, the four constructs have coding sequences derived from either MTY811c or Tyrs-J. TyBS contains an *XhoI-EcoRI* fragment from clone Tyrs-J; it encodes cysteine at amino acid 103 and glycine at amino acid 346. Ty811B contains the corresponding *XhoI-EcoRI* fragment from MTY811c encoding serine at amino acid 103 and valine at amino acid 346. Ty811C and Ty811D are chimeric constructs, each containing one end from Tyrs-J and the other end from MTY811c, ligated at a central *ScaI* site (see Fig. 1). Ty811C codes for a cysteine at amino acid 103, while TY811D codes for a serine.

Each construct was isolated as a *KpnI-EcoRI* fragment and microinjected into one-cell stage fertilized FVB/N (albino) embryos. Embryos that survived injection were implanted into pseudopregnant ICR (albino) females and the offspring were screened for integration of the tyrosinase minigenes by Southern hybridizations to tail DNAs (Fig. 2, summary in Table 1). Head-to-tail integration of the microinjected DNA yields a diagnostic hybridization band at 4.1 kb after digestion with *PstI* (Fig. 2).

All nine mice that were transgenic for Ty811B or Ty811D were albino (Table 1). By contrast, the TyBS and Ty811C constructs each produced pigmented transgenic mice (Table 1). The only difference between TyBS and Ty811D, and similarly between Ty811C and Ty811B is a cysteine to serine change at amino acid 103.
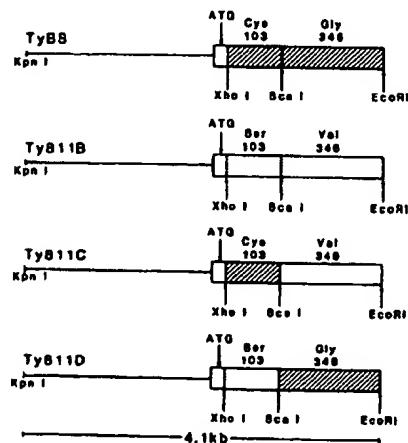

Fig. 1. Tyrosinase minigene constructs (TyBS, Ty811B, Ty811C and Ty811D). All four constructs have an identical 2.25 kb tyrosinase promoter (single line) plus 65 bp of tyrosinase exon 1 (to the *XhoI* site). The cDNA regions are derived from MTY811c (open box) or from Tyrs-J (shaded).

Pigmented transgenic mice

FVB/N mice are an inbred strain of mice that are homozygous mutants at the albino locus, but are wild-type at the agouti, brown and dilute loci (P. A. Overbeek, unpublished). Full expression of a functional tyrosinase minigene would be expected to convert the albino phenotype to an agouti pigmentation. However, the transgenic mice obtained with both the TyBS and Ty811C constructs showed considerable variation in their pigmentation. The coat colors were found to range from grayish (similar to the pigmentation of chinchilla (c^{ch}) or dilute (d) mutants) to brownish (see Fig. 3). None of the mice were black. Similarly, the intensity of the pigmentation varied from barely visible to near normal (Fig. 3A and 3C). One TyBS mouse and four Ty811C mice were mottled (Fig. 3A and 3C). All four TyBS transgenic mice and eight out of the 14 Ty811C transgenic mice had dark eyes at birth and were immediately identifiable as transgenic mice. Three other Ty811C mice showed light pigmentation in both fur and eyes by 14 days of age (Fig. 3C, mice 4,5, & 6). None of the initial TyBS or Ty811C mice showed normal agouti pigmentation. However, pigmented male and female offspring of the mottled TyBS female show normal agouti pigmentation (Fig. 3B), implying that the founder mouse is mosaic and also that the TyBS minigene is sufficient to produce normal agouti pigmentation.

A conserved mutation in albino mouse strains

Our tyrosinase minigene study suggests that a G to C mutation at nucleotide 308 is sufficient to inactivate the tyrosinase gene. To determine whether a similar mutation is present in other albino mouse strains, the corresponding region of the genome was sequenced for a collection of pigmented and albino mouse strains. The albino strains were selected to include inbred and outbred mice of various origins. The first exon of the tyrosinase gene was amplified using the polymerase chain reaction (PCR) (19). The region surrounding nucleotide 308 was then sequenced directly by use of a ^{32}P end-labelled internal primer (Fig. 4A). All of the classical albino strains examined (see Table 2) were found to have a C rather than a G at nucleotide 308 of the coding strand of tyrosinase (for example, see Fig. 4B). By comparison, all pigmented mouse strains examined (Table 2) have a G at this position. Both the c/c and c/c^{ch} variants of strain 129/J were also sequenced (Fig. 4C). Heterozygous c/c^{ch} mice show both C and G nucleotides at position 308, while homozygous c/c mice encode only serine. The G to C transversion exhibits 100% concordance with the classical albino phenotype.

Mouse strain CE/J, which has the c^e (extreme dilute) mutation at the albino locus, encodes a cysteine at amino acid 103 (Fig. 4C), indicating that the c^e mutation lies elsewhere in the tyrosinase gene. We also sequenced the more recently established
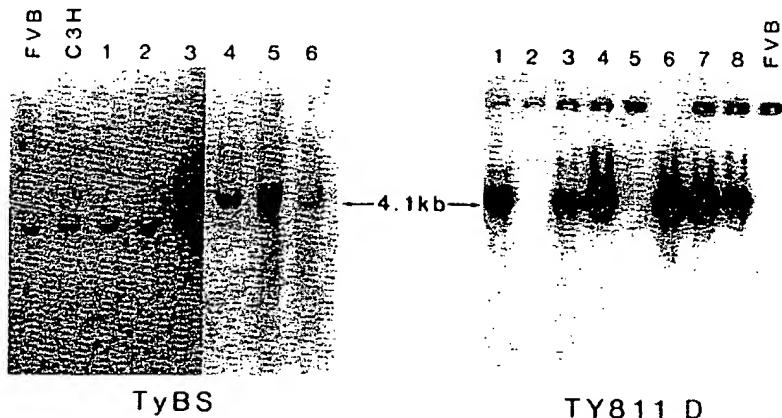

TyBS

TY811 D

Fig. 2. Southern hybridization analysis of tail DNAs from TyBS and Ty811D founder mice. Tail DNAs were digested with *Pst*I, fractionated by agarose gel electrophoresis, transferred to a Zeta-probe membrane and hybridized with a tyrosinase cDNA probe. The tyrosinase minigenes have a single *Pst*I restriction site. DNAs from the first six newborns injected with TyBS are shown in the left panel; DNAs from the eight newborns injected with Ty811D are shown on the right. FVB and C3H: albino and pigmented non-transgenic control mice. TyBS mice: lanes 1 and 2, DNAs from albino mice; lanes 3–6, DNAs from pigmented mice. All of the Ty811D mice were albino. Exposure time for TyBS lanes 4 to 6 was 3 hours. All other lanes were exposed for 18 hours.

Table 1.

Tyrosinase minigene construct	No. of newborn mice	No. of transgenic mice	No. of pigmented mice
TyBS	6	4	4
Ty811B	23	3	0
Ty811C	39	14	11
Ty811D	8	6	0

Pigmentation in transgenic mice generated with the different tyrosinase minigenes. Injections of Ty811B and Ty811D generated a total of 9 transgenic founder animals, none of which were pigmented. Injections of TyBS and Ty811C yielded 18 transgenic mice, 15 of which (83%) were pigmented.
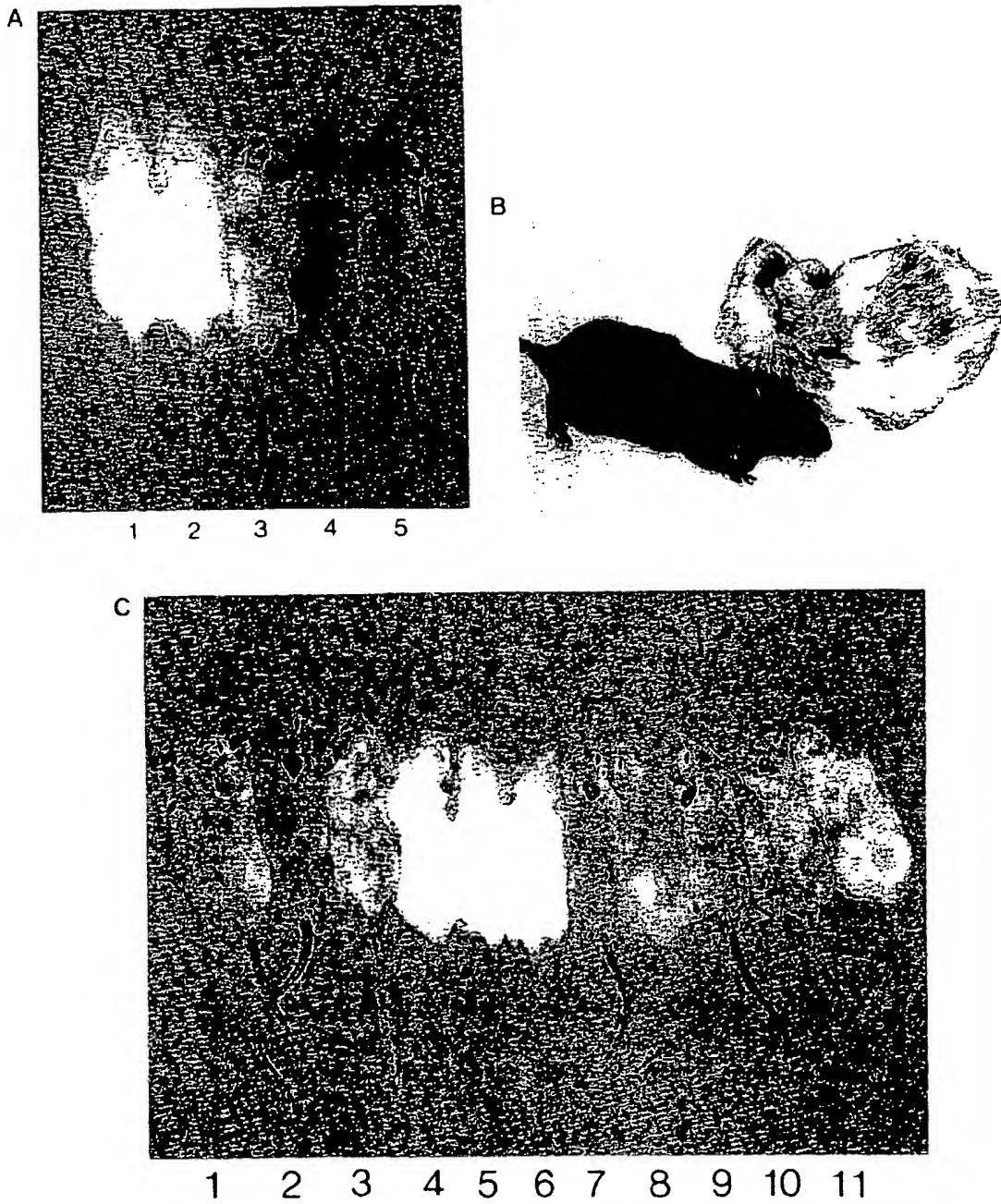
Fig. 3. Photographs of pigmented TyBS and Ty811C mice. A. TyBS transgenic mice. Mice 1–5 correspond to Fig. 2, TyBS lanes 1–5. Two mice (4 and 5) show a grayish coat color. One mouse (#3) is mottled. B. The mottled TyBS founder mouse (panel A, mouse #3) and one of its offspring. C. Ty811C pigmented founder mice. One mouse (#2) shows a grayish coat color. Three mice (1, 7, and 9) have brownish fur. Three mice (4, 5, and 6) show light pigmentation. One of these mice (#4) has darkly pigmented ears and tail similar to himalayan (c^h) mutants. Four mice (3, 8, 10 and 11) are mottled.

albino mouse strains C3H/HeJ-c^{9J}, CBA/N-c^{10J}, and C57BL/10SsJ-c^{11J}. These strains all have a G at nucleotide 308 (Table 2) indicating that their mutations lie elsewhere in the tyrosinase gene. The c^{11J} strain was found to have an A rather than a G at nucleotide 230, resulting in an Arg to Leu change at amino acid 77.

A

B

C
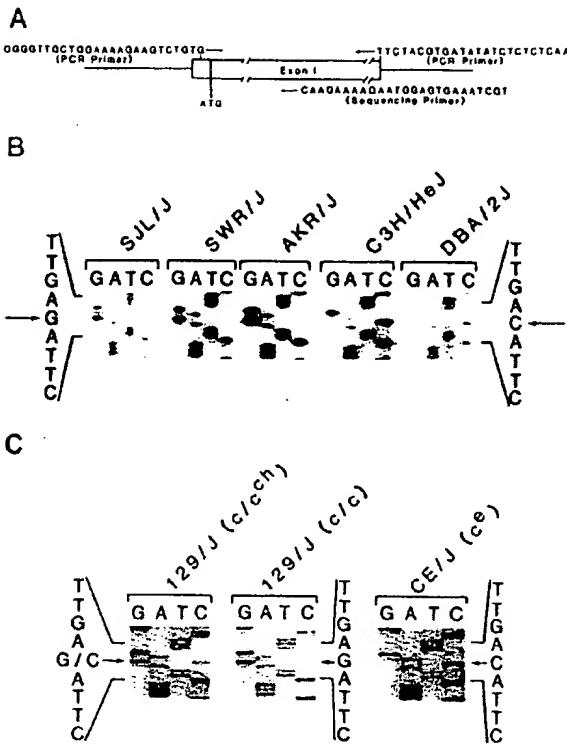
Fig. 4. A. Sequencing of the classical albino mutation. Schematic of PCR amplification and sequencing. Two oligonucleotide primers were designed to amplify the whole coding region of tyrosinase exon 1 by PCR. Sequencing was performed using an internal primer with an antisense orientation. B. Nucleotide sequences of albino (SJL/J, SWR/J and AKR/J) and pigmented (C3H/HeJ and DBA/2J) mouse strains. The antisense sequences for the nucleotides that encode amino acids 102–104 are shown. The corresponding coding sequences are AA-CTGTAAG (asn cys lys) for the pigmented strains and AACTCTAAG (asn ser lys) for the albino strains. The coding sequence G to C transversion is located at nucleotide 308. The arrow indicates the site of the conserved mutation. C. Nucleotide sequences of other c-locus mutants. 129/J c/c is homozygous for the classical albino mutation. 129/J c/c^ch^ is heterozygous for the albino and chinchilla mutations and shows both C and G bands at nucleotide 308. The c^e^ (extreme dilution) mutation in strain CE/J does not alter the cysteine at amino acid 103.

DISCUSSION

Classical albino mutation

The ability of tyrosinase minigenes to induce pigmentation in transgenic mice has been reported previously (13,14). The previous tyrosinase constructs encoded a cysteine at amino acid 103 and were linked to regulatory sequences from the genomes of pigmented mouse strains (C57BL/6 and 129/J respectively). Our tyrosinase minigenes were linked to 5' upstream regulatory sequences from the genome of an albino (BALB/c) strain of mice. Both the TyBS and Ty811C constructs produced pigmented transgenic mice, implying that the BALB/c promoter region is functional and that albinism in BALB/c mice is not due to a defective promoter. The minigenes TyBS, Ty811B, Ty811C and Ty811D were constructed to test whether specific point mutations within the coding sequences of tyrosinase could inactivate the gene. The absence of pigmentation in mice transgenic for Ty811B

Table 2. Coat color and nucleotide 308 comparison in pigmented and albino mouse strains.

Mouse strain	Coat color	Nucleotide 308 (sense strand)
Pigmented strains		
AU/SsJ	Black	G
C3H/HeJ	Agouti	G
C57BL/6J	Black	G
C57BR/cdJ	Brown	G
C57L/J	Leaden	G
C58/J	Black	G
CBA/J	Agouti	G
DBA/2J	Dilute brown	G
NZB/B1NJ	Black	G
P/J	Pink-eyed fawn	G
SM/J	Light-bellied Agouti	G
WB/ReJ-W	Black	G
Classical albino strains		
A/HeJ	Albino	C
AKR/J	Albino	C
BALB/cByJ	Albino	C
FVB/N	Albino	C
NZW/LacJ	Albino	C
SJL/J	Albino	C
SWR/J	Albino	C
129/J	Albino (c/c)	C
	Chinchilla (c/c^ch^)	C and G
ICR/Hsd*	Albino	C
Other strains		
C3H/HeJ-c^9J^	Albino [+]	G
CBA/N-c^10J^	Albino [+]	G
C57BL/10SnJ-c^11J^	Albino [+]	G
CE/J (c^R^)	Extreme dilute	G

* Outbred strain
[+] Albino mutations identified in pigmented strains of mice

and Ty811D might be due to: 1) lack of expression of the transgenes, 2) artifacts introduced during the cloning procedure, or 3) inactivation of the tyrosinase genes by the cysteine to serine mutations. Our tyrosinase minigenes were constructed to give transcripts that were identical to the endogenous transcripts in order to eliminate any artifacts due to inappropriate sequences. We have not tried to document directly Ty811B or Ty811D expression. However, since the two constructs differ by only single nucleotides from TyBS and Ty811C, the frequencies of expression are expected to be equivalent. Over 80% of the founder mice that carry TyBS or Ty811C show expression and pigmentation. Thus, we feel that the lack of pigmentation in all nine Ty811B and Ty811D families is not likely to be due to lack of expression in every family. In order to check for cloning artifacts, important regions of Ty811B and Ty811D were sequenced. The only base pair changes detected were the G to C mutations at nucleotide 308. Thus, the most likely explanation for the absence of pigmentation in the mice transgenic for Ty811B and Ty811D is that the cysteine to serine changes at amino acid 103 are sufficient to inactivate the tyrosinase minigenes.

The region of the genome that encodes cysteine 103 was sequenced in a variety of pigmented and albino strains of mice (Table 2). All of the classical albino strains showed an identical G to C transversion at nucleotide 308, resulting in 100% concordance between classical albinism and the presence of a serine at amino acid 103. Our experimental results are fully consistent with the proposal that a missense mutation at amino

acid 103 is the cause of the classical albino mutation in laboratory mice.

Tyrosinase promoter

Our tyrosinase minigenes contain 2.25 kb of 5' upstream regulatory sequences. This is a shorter regulatory region than that used by either Tanaka et al. (13) or Beerman et al. (14). The shorter promoter can function at most sites of integration in the genome (83% pigmentation in the TyBS and Ty811C founder mice) and can be sufficiently active to yield full agouti pigmentation (Fig. 3B). Integration of a high number of copies of the tyrosinase minigene is not required to give agouti pigmentation. The founder mouse for the fully pigmented family had the lowest copy number of the original set of TyBS transgenic mice (Fig. 2), and the fully pigmented offspring (Fig. 3B) carry only 2–4 copies of TyBS per genome (data not shown). The tyrosinase promoter can function in both melanocytes (neural crest origin) and pigment epithelial cells of the retina (neuroectodermal origin). In addition, the promoter appears sufficient to provide appropriate developmental regulation of gene expression, including induction of pigmentation in the retinal pigment epithelial cells by day 13 of embryonic development (data not shown).

Coat color

Mice that are transgenic for the tyrosinase minigenes TyBS and Ty811C show considerable variation in their pigmentation. We have generated over 50 additional pigmented transgenic families with the TyBS construct, and the additional families show the same variability of pigmentation (data not shown). Some of the families show full agouti pigmentation (e.g. Fig. 3B), implying that the tyrosinase minigene is sufficient to produce normal levels of both eumelanin and pheomelanin. Further mating studies have revealed that the tyrosinase minigene can give normal black or brown pigmentation on the appropriate non-agouti genetic backgrounds (P. A. Overbeek, personal observation). Less intense pigmentation is seen in most families. Of particular interest is the fact that some families show pigmentation similar to chinchilla (c^{ch}) mutants, while other families have dark ears and light bodies, a phenotype also produced by the himalayan (c^h) mutation. The chinchilla mutation lies in the tyrosinase coding sequences at amino acid 464 (14). Himalayan mutants have a temperature sensitive tyrosinase and have been reported previously to have a His to Arg amino acid change at position 420 (20). For the transgenic mice, the variable patterns of pigmentation are likely to reflect different levels of transgene expression from different sites of integration in the genome. Considered together, these observations suggest that nearly identical mutant pigmentation patterns can occur as a result of alterations in either the coding sequences, or the transcriptional regulation, of the tyrosinase gene. About 15% of the families transgenic for TyBS or Ty811C do not show pigmentation, perhaps due to rearrangements of the transgenic DNA during integration or integration into inactive regions of the genome.

Common ancestor

The classical albino strains of mice that we examined included both inbred and outbred strains. All had an identical G to C transversion at nucleotide 308. In contrast, the more recently identified albino mutations c^{9J}, c^{10J}, and c^{11J} did not show the same mutation. The c^{11J} albinism may be due to an Arg to Leu change at amino acid 77. These sequencing results imply that mutations other than the G to C transversion at nucleotide 308

can cause albinism. Since the classical albino strains of mice all exhibit the same G to C transversion (Table 2) and the same *Eco*RI haplotype (data not shown), the data imply that these mice are all derived from a common ancestor. Although classical albino strains of laboratory mice were derived by a variety of investigators at different times and different locations (17), they all appear to be offspring of a single albino ancestor.

Jackson and Bennett (21) have reached similar conclusions based on their studies of pigmented revertants of a cultured line of albino melanocytes and studies of a *Dde*I polymorphism at nucleotide 308.

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